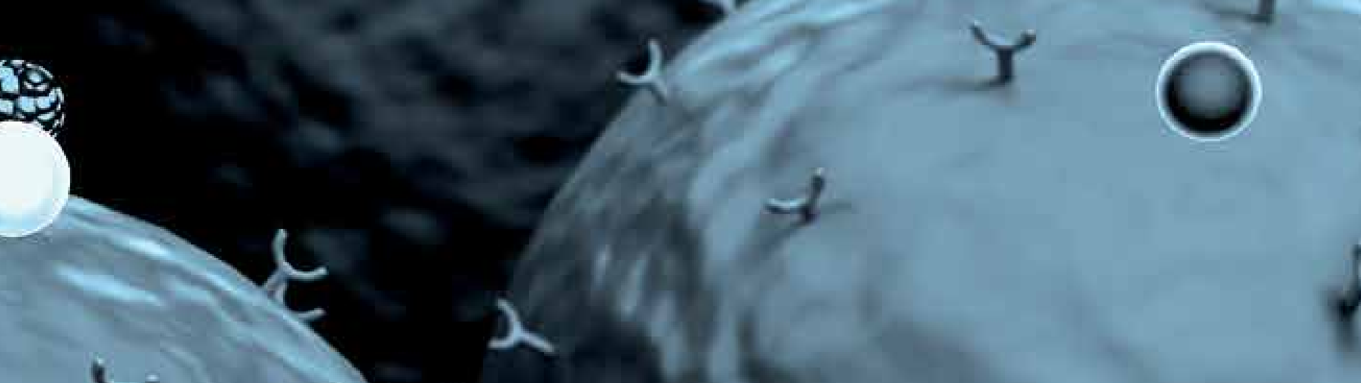

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# Cell Death
## Autophagy, Apoptosis and Necrosis

*Edited by Tobias M. Ntuli*

# CELL DEATH - AUTOPHAGY, APOPTOSIS AND NECROSIS

Edited by **Tobias M. Ntuli**

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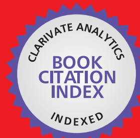


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Dr. Tobias M. Ntuli holds a doctorate in Cell Biology from the Department of Biological and Conservation Sciences at the University of KwaZulu-Natal in Durban, South Africa. Dr. Ntuli has carried out numerous postdoctoral research on various universities such as the Universty of Cape Town, South Africa, Tropical Botanical Gardens in Menglun, Mengla, Yunnan in China and at the Univeristy of South Africa based in Structural Biology Laboratory of the Biosciences Unit of the Council of Scientific and Industrial Research in Petoria. He edited a book entitled "Apoptosis and Medicine" in 2012. He contributed a chapter entitled "Drought and Desiccation-Tolerance and Sensitivity in Plants". Dr. Ntuli's current research interests include anti-oxidants, apostosis, programmed cell death, espiratory metabolism, free radical activity, ipid peroxidation and metabolic dysunction, regulation and imbalance.

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# Preface

Autophagy is a genetic program that secures the survival of eukaryotic cells to compensate for periods of starvation and cellular stress. Apoptosis, in contrast, is a genetically defined program leading to cell death. Both pathways are interconnected through conserved co-regulatory signaling pathways that are context-dependent. Proper co-regulation of autophagy and apoptosis, whereby autophagy exerts an anti-apoptotic function and apoptosis inhibits autophagic survival strategies, critically secures the survival of healthy cells and counteract genomic instability in eukaryotic organisms. Necrosis is a form of cell injury which results in the premature deathof cells in living tissue by autolysis.

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Section 1

# Autophagy

Chapter 1

# Autophagy in Cell Fate and Diseases

Daniel Grasso, Alejandro Ropolo and Maria I. Vaccaro

Additional information is available at the end of the chapter

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**Abstract**

Autophagy pathway has been one of the hot topics during the last decade. From a general notion about its cellular role, autophagy becomes a more sophisticated phenomenon with significant implications in cellular homeostasis. Consequently, autophagy represents an emerging new factor in human diseases. Despite its general task, the bulk degradation of cellular constituents during starvation settings, autophagy possesses important cross talk and interrelationships with several cellular processes such as apoptosis and senescence, among others. This entire panorama gives us a complex but exciting scenario. Consequently, with the aim of encompassing the whole spectrum, in this chapter, we review three main topics: autophagy as a cellular process; autophagy in cell fate; and autophagy in disease. We discuss the emerging role of selective type of autophagy to avoid apoptosis or necrosis and the novel relationship between autophagy and senescence to understand the real extent that autophagy pathway has over cell fate. Finally, we briefly describe the current trends on autophagy in human pancreatic diseases and its role in cancer cell metabolism.

**Keywords:** VMP1, zymophagy, senescence, acute pancreatitis, pancreatic cancer

## 1. Introduction

Autophagy is a highly regulated cellular pathway for degrading long-lived proteins and is the only known pathway for clearing cytoplasmic organelles. Autophagy is a major contributor to maintain cellular homeostasis and metabolism.

Autophagy is an evolutionarily conserved and highly regulated lysosomal pathway that degrades macromolecules (e.g., proteins, glycogen, lipids, and nucleotides) and cytoplasmic

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organelles [1-3]. This catabolic process is involved in the turnover of long-lived proteins and other cellular macromolecules and it might play a protective role in development, aging, cell death, and defense against intracellular pathogens [4,5]. Moreover, autophagy has been linked to a variety of pathological processes such as neurodegenerative diseases and tumorigenesis, which highlights its biological and medical importance [6,7].

Although autophagy was first identified in mammalian liver upon glucagon treatment approximately 50 years ago, its molecular understanding began only in the past decade, largely based on the discovery of the autophagy-related genes (ATGs) by genetic analyses in yeast. Since the discovery of the yeast ATG proteins, autophagosome formation has been dissected at the molecular level, but a lot of questions about this pathway remain unanswered. In mammalian cells, the sequential association of at least a subset of the ATG proteins leads to the assembly of the pre-autophagosome structure (PAS). PAS formation also requires PtdIns3P generation and it is thought that this lipid is present in specialized subdomains of membranes where the PAS is assembled and autophagosomes are generated.

One of the autophagy-related proteins is VACUOLE MEMBRANE PROTEIN 1 (VMP1), whose expression triggers autophagy in mammalian cells even under nutrient-rich conditions. Conversely, autophagy is completely blocked in the absence of VMP1. VMP1 is required for the biogenesis of autophagosomes in mammalian cells in all conditions underscoring its upstream regulatory function in autophagy. Importantly, VMP1 is also expressed early during the onset of several pathologies including diabetes mellitus, pancreatitis, and pancreatic cancer.

The presence of autophagy has been described in dying acinar cells in tissue from human diseases. Though controversy exists about whether autophagy could be at the same time a survival cell response and a programmed cell death pathway, there is no discussion that autophagy has the power to change the fate of a cell. From an energetic homeostasis point of view, autophagy is a major cellular response against starvation condition and plays key roles in other cellular stress conditions such as oxidative damage. In those cases, autophagy is not only a way to keep energy and nutrition but also a specific mechanism of cell adaptive response.

Increasing evidence suggests that autophagy may influence the pathogenesis of human diseases including cancer, neurodegenerative diseases, inflammatory diseases, and metabolic diseases. Several inherited myopathies are associated with aberrant autophagy. Recent investigations have explored the functions of autophagy in the pathogenesis of metabolic disorders such as diabetes, insulin resistance, and obesity, and the progression of aging.

It is possible to think that different types of autophagy may be involved in the initial cellular events induced by the noxa. Autophagic processes might be triggered in different cells by several diseases and probably they are more complex than we actually understand. These pathways function as adaptive responses that mostly act as protective mechanisms against cell healing. The knowledge of the molecular mechanisms of a sophisticated membrane transport system such as autophagy would provide bases for novel and more rational diagnostic and therapeutic strategies.

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## 2. Autophagy, a complex cell event

Autophagy consists of several sequential steps: induction, autophagosome formation, autophagosome-lysosome fusion, and degradation. Depending on the delivery route of the cytoplasmic material to the lysosome, there are three major types of autophagy in eukaryotes: 1) chaperone-mediated autophagy (CMA), 2) microautophagy, and 3) macroautophagy, hereafter referred to as autophagy [8]. CMA allows the direct lysosomal import of unfolded soluble proteins that contain a particular pentapeptide motif. In microautophagy, cytoplasmic material is directly engulfed into the lysosome at the surface of the lysosome by membrane rearrangement. Finally, autophagy involves the sequestration of cytoplasm into a double-membrane cytosolic vesicle referred to as an autophagosome that subsequently fuses with a lysosome to form an autolysosome for degradation by lysosomal hydrolases [9].

### 2.1. The autophagic process

Autophagy is characterized by sequestration of bulk cytoplasm and organelles in double-membrane vesicles called autophagosomes that eventually acquire lysosomal-like features [9,10]. The autophagic process is described in Figure 1.

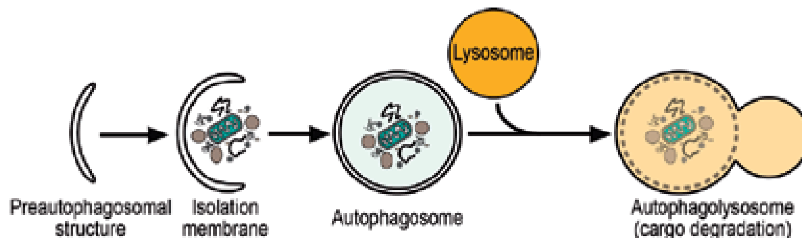


**Figure 1.** General scheme of autophagic process. During autophagy an isolation membrane forms, invaginates and sequesters cytoplasmic constituents. The edges of the membrane fuse to form the autophagosome. The outer membrane of the autophagosome fuses with the lysosome to form the autolysosome, where the cargo is degraded.

Briefly, sequestration of cytoplasm into a double-membrane cytosolic vesicle is followed by the fusion of the vesicle with a late endosome or lysosome to form an autophagolysosome (or autolysosome). Then, inner membrane of the autophagosome and autophagosome-containing cytoplasm-derived materials are degraded by lysosomal/vacuolar hydrolases inside the autophagosome. The molecular mechanisms underlying the transport and fusion of autophagosomes are just beginning to be understood and through active investigations, several major events involved in the process have recently been clarified including the recycling of lysosomes [11]. In mammalian cells, autophagosome maturation is a prior step for the fusion between autophagosomes and lysosomes. The degradation products, including macromolecules, are then exported to the cytosol for reuse by the cell.

### 2.2. The autophagosome at a molecular level

Since the discovery of yeast ATG proteins, autophagosome formation has been dissected at the molecular level but a lot of questions about the molecular mechanism underlying this

process remain unanswered. Autophagosomes can be considered unique organelles because they do not contain marker proteins of other subcellular compartments [12]. In mammalian cells, the sequential association of at least a subset of the ATG proteins leads to the assembly of the PAS that is believed to be the site where the precursor structure of the autophagosomes, the phagophores, are generated [13]. The PAS and phagophore formation also requires phosphatidylinositol 3-phosphate (PI3P) [14] and it is believed to be associated to specific subdomains of the endoplasmic reticulum (ER), termed omegasomes [15,16]. Among the key mediators initiating autophagosome formation, there is a set of evolutionarily conserved ATG gene products: the kinase-containing Ulk1/2 complex (ATG1 in yeast), the Class III phosphatidylinositol 3-kinase (PI3K) complex (composed by BECN1/ATG6-hVps34, hVps15 and ATG14L), the ubiquitin-like conjugation systems leading to the formation of the ATG5–ATG12–ATG16L1 complex, and the LC3/ATG8 phosphatidylethanolamine-conjugate (e.g., LC3-II) [17]. A second group of ATG proteins, which does not have orthologous group in yeast, has also recently emerged and appears to play a key role in regulating autophagy in high eukaryotes. One of these proteins is the transmembrane protein VMP1, whose expression triggers autophagy in mammalian cells even under nutrient-rich conditions [18,19]. Conversely, autophagy is completely blocked in the absence of VMP1 [18].

The autophagosome formation process is composed of isolation membrane nucleation, elongation, and completion steps. In mammals, the Class III PI3K plays an essential role in isolation membrane nucleation during autophagy [20]. The Class III PI3K is associated with BECN1/ATG6 and p150, the homolog of Vps15 (phosphoinositide-3- kinase regulatory subunit 4), to form the PI3K complex. This kinase catalyzes the generation of PI3P on the autophagosomal membrane, favoring the localization of other ATG proteins to the PAS during autophagosomal formation. The autophagosome nucleation system is ATG12-ATG5-ATG16L, which is a ubiquitin-like protein conjugation system essential for the formation of the PAS. ATG12 is conjugated to ATG5 [21]. E1-like ATG7 activates the carboxyl-terminal glycine residue of ATG12 through a high-energy thioester bond in an ATP-dependent manner. The ATG12-ATG5 conjugate further interacts with ATG16L1 to form a ~350 kDa multimeric ATG12-ATG5-ATG16L protein complex through the homo-oligomerization of ATG16L [22]. Another ubiquitin-like protein conjugation system is the modification of LC3 (a mammalian homolog of ATG8) by the phospholipid phosphatidylethanolamine (PE) [22], an essential process for the formation of autophagosomes. The cytosolic form of LC3 (LC3-I) is cleaved by cysteine protease ATG4 and then conjugated with PE by ATG7 and ATG3. This lipidated LC3 (LC3-II) then associates with newly forming autophagosome membranes. LC3-II remains on mature autophagosomes until its fusion with lysosomes [23, 24]. The conversion of LC3-I to LC3-II is thus well known as a marker of autophagy (Figure 2). However, the increase of LC3-II alone is not enough to show autophagy activation because the inhibition of LC3-II degradation in the lysosome by the impaired autophagy flux can also cause its accumulation.

While the origin of autophagic vacuoles remains disputable, several hypotheses have been proposed for the source of autophagosomal membrane during autophagosome formation. The first hypothesis is “de novo” formation of autophagosome by ATG9 reservoirs. In the second hypothesis, various organelles such as ER, mitochondria, and plasma membrane are used as

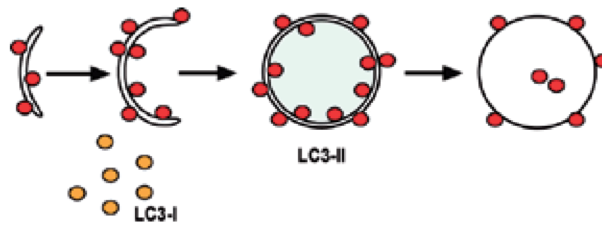


**Figure 2.** During autophagy the cytosolic form of LC3 (LC3-I) undergoes C-terminal proteolysis and lipidation (LC3-II) and translocates to the autophagosomal membrane. LC3 is currently used as a specific marker of autophagy.

an origin for the formation of the phagophore. Recently, cup-shaped structures called omegasome, a discrete region of the ER, were identified as a platform for autophagosome formation [25]. The ATG5 complex, LC3, and ULK1 have been shown to recruit into the omegasome after starvation, and ATG5- and LC3-positive membranes seem to emerge from the omegasome. It was also observed that omegasomes form in close proximity to the PI3K-containing vesicles which may synthesize the PI3P. This hypothesis is also supported by a notion of a physical association between the ER and early autophagic membranes [26].

### 2.3. Autophagy induction

Basal autophagy in unstressed cells is kept down by the action of the mammalian target of rapamycin complex 1 (mTORC1). Key upstream regulators of mTORC1 include the class I phosphoinositide 3-kinase-Akt pathway which keeps mTORC1 active in cells with sufficient growth factors and the AMP-activated protein kinase (AMPK) pathway that inhibits mTORC1 upon starvation and calcium signals [27,28].

Under stress conditions such as amino acid starvation, autophagy is strongly induced in many types of cultured cells. The effects of individual amino acids differ in their abilities to regulate autophagy. Amino acids including Leu, Tyr, Phe, Gln, Pro, His, Trp, Met, and Ala suppress autophagy in ex vivo perfused liver [29]. However, such profiles depend on cell types showing their different amino acid metabolisms in tissues. The questions on how cells sense amino acid concentration and physiological significance of autophagy regulation by amino acid starvation are not fully understood yet. It has been demonstrated that amino acid signaling pathways exist, which involve activation of mTORC1 and the subsequent regulation of the Class III PI3K. mTORC1 is involved in the control of multiple cell processes in response to changes in nutrient conditions [30]. Especially, mTORC1 requires Rag GTPase, Rheb, and Vps34 for its activation and subsequent inhibition of autophagy in response to amino acids [31, 32]. Energy levels are primarily sensed by AMPK, a key factor for cellular energy homeostasis. In low energy states, AMPK is activated and the activated AMPK then inactivates mTORC1 through TSC1/TSC2 and Rheb protein [33].

Thus, inactivation of mTORC1 is essential for the induction of autophagy and plays a central role in autophagy. In addition to amino acid signaling, hormones, growth factors, and many other factors including Bcl-2 [34] have also been reported to regulate autophagy. But, not all autophagy signals are transduced through mTOR signaling. A recent study showed that small-

molecule enhancers of the cytostatic effects of rapamycin (called SMERs) induce autophagy independently of mTOR [35]. Activities of the ULK1 are regulated by mTOR depending on nutrient conditions. Under growing and high-nutrient conditions, active mTORC1 interacts with ULK1 and phosphorylates ULK1 and mATG13 and, thus, inhibits the membrane targeting of the ULK1. During starvation condition, on the other hand, inactivated mTORC1 dissociates from ULK1 and results in the ULK1 complex formation (ULK1-mATG13-FIP200-ATG101) leading to autophagy induction [36].

### 2.4. The autophagy-related protein Beclin 1 (BECN1)

BECN1 (former Beclin 1), the mammalian ATG6, is a haploinsufficient tumor suppressor and an important effector of autophagy. BECN1 is a subunit of the PI3K complex, the action of which is antagonized by Bcl-2 [36,37]. BECN1 contains a BH3 domain that mediates its interaction with Bcl-2 [38,39]. The interaction between Bcl-2 and BECN1 leads to inhibition of autophagy by interfering with the formation and activity of the autophagy promoter complex, BECN1- PI3K [40] (Figure 3).

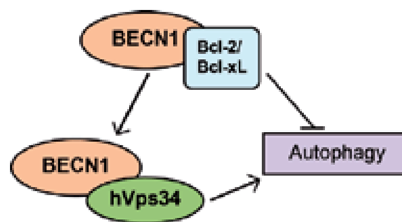


**Figure 3.** BECN1 is an effector of autophagy whose interaction with Bcl-2 inhibits its role as autophagy promoter. BECN1 and the PI3K complex, where hVP34 is the active subunit, are necessary for PI3P production over the autophagic structures and the consequent recruitment of additional ATG proteins.

### 2.5. The Vacuole Membrane Protein 1 (VMP1)

The pancreatitis-associated protein named VMP1 is a transmembrane protein with no known homologs in yeast. VMP1 was found searching for new molecules that were differentially expressed during acute pancreatitis [41]. VMP1 expression is induced by mutated K-Ras in pancreatic cancer cells [42] and by hyperstimulation of Gq-coupled cholecystokinin receptor (CCK-R) in pancreatic acinar cells during acute pancreatitis [43]. In the adult normal pancreas, VMP1 expression is not detectable but it is highly induced early during experimental acute pancreatitis and its expression levels correlate with morphological features resembling autophagy [44]. Moreover, VMP1 expression can be found in pancreatic acinar cells from rats developing spontaneous chronic pancreatitis (WBN/Kob rats) [45] and it is rapidly and highly expressed in experimental diabetes [45-47]. Finally, gemcitabine (2,2-difluorodeoxycytidine), the standard chemotherapy for the treatment of advanced pancreatic cancer, induces VMP1 expression in human pancreatic cancer cells [48-50]. We demonstrated that VMP1 expression triggers autophagosome formation in mammalian cells even under nutrient-replete conditions [18,19]. Remarkably, VMP1 pancreas-specific transgenic expression in mice promotes auto-

phagosome formation in acinar cells. Therefore, VMP1 expression may be involved in autophagy induction during acute pancreatitis, a disease defined as pancreas self-digestion. Furthermore, VMP1 is the only human-disease-inducible ATG-protein described so far.

VMP1 interacts with BECN1 through its hydrophilic C-terminal region (VMP1-ATGD) that is necessary for early steps of autophagosome formation. Hierarchical analyses in mammalian cells shows that VMP1 along with ULK1 localizes in the autophagosome formation site [13]. VMP1-BECN1 interaction is required for the formation of the PI3K complex acting in mammalian autophagy. This complex, which is composed of BECN1-hVps34-ATG14, promotes PI3P generation on autophagosomal membrane, favoring the localization of ATG16L1 and LC3 to the autophagosomal membrane during autophagosome formation [51,52]. The interaction between VMP1 and BECN1 requires the BECN1 domain that binds to Bcl-2 (BECN1-BH3 domain) [51]. During normal growth conditions, Bcl-2 binding to BECN1 is maximal, and when autophagy is induced, this interaction is strongly reduced [53]. VMP1 expression leads to the dissolution of the BECN1-Bcl-2 complex, indicating that VMP1 is involved in driving BECN1 into the autophagic process [38]. Thus, VMP1-BECN1 interaction through the VMP1-ATGD is required for the proper localization of PI3K activity on the autophagosomal membrane during mammalian autophagy, positioning VMP1 as a key regulator of the early steps of autophagosome formation possibly acting as a platform in the autophagosomal membrane.

### 2.6. Autophagy, a selective process

Early studies suggested that autophagy was a nonselective process in which cytoplasmic structures were randomly sequestered into autophagosomes before being delivered to the mammalian lysosome or the plant and yeast vacuole for degradation. Now, there is growing evidence that unwanted cellular structures can be selectively recognized and exclusively eliminated within cells. This is achieved through the action of specific autophagy receptors such as Nbr1 and p62, which is a ubiquitin-binding protein that interacts with LC3 [10,54]. Thus, excess or damaged organelles, including mitochondria, peroxisomes, lipid droplets, endoplasmic reticulum, and ribosomes, can be specifically sequestered by autophagosomes and targeted to the lysosome for degradation. Importantly, there is growing evidence that selective autophagy subtypes also have a wide range of physiological functions. Selective autophagic pathways target distinct cargoes to autophagosomes including mechanisms for the clearance of aggregated protein and for the removal of dysfunctional mitochondria (mitophagy). In pancreatic cells, autophagy has recently been shown to specifically turn over secretory granules damaged by acute pancreatitis as a protective cellular response [43].

### 2.7. Zymophagy, a novel selective type of autophagy

The pancreatic acinar cell activates VMP1-mediated autophagy early during acute pancreatitis [44]. Relevant data about the role of autophagy in pancreas were obtained using ElaI-VMP1 mice in which the pancreaatic acinar-cell-specific elastase promoter drives VMP1 expression in pancreas. Pancreases of these transgenic mice show numerous vesicles that stain for endogenous LC3, indicating that VMP1 induces the autophagosome formation and, therefore,

autophagy [18]. Interestingly, ElaI-VMP1 mice do not develop pancreatitis in normal conditions, confirming that autophagosome formation does not induce acute pancreatitis [18]. The immunomagnetic isolation of VMP1-autophagosomes containing zymogen granules from the ElaI-VMP1 transgenic mouse pancreas with acute pancreatitis allowed the discovery of a new type of selective autophagy named zymophagy that functions as an inducible cellular process that recognizes and degrades activated zymogen granules [43].

Zymophagy is characterized by the formation of autophagosomes containing zymogen granules. These organelles mediate the sequestration and degradation of pancreatitis-activated zymogen granules. CCK-R hyperstimulation with cerulein in wild-type animals, a classical model of acute pancreatitis, induced a markedly altered distribution pattern of the secretory granules such as fusion among zymogen granules as well as their fusion with condensing vacuoles. In addition, acinar cells lose their polarity, which results in the relocation of zymogen granules to the basolateral membrane. Surprisingly, ElaI-VMP1 mice subjected to CCK-R hyperstimulation reveal that acinar cells preserve their structure and polarity with negligible or no alteration in vesicular transport. Instead, pancreases from cerulein-treated ElaI-VMP1 mice presented autophagosomes containing zymogen granules displaying a distinct localization to the apical area of the acinar cell. This observation is confirmed using isolated mouse pancreas acini revealing that 15 min after cerulein treatment, zymophagy is detected [43]. The finding of different maturation levels of selective autophagic vesicles as well as the degradation of p62 provide evidence that autophagic flow remains primarily unchanged under CCK-R hyperstimulation [43].

Regarding the pathophysiological relevance of zymophagy during acute pancreatitis, it was demonstrated that zymophagy protects acinar cells from intracellular trypsinogen activation triggered in vivo by experimental pancreatitis induced by cerulein. Upon CCK-R hyperstimulation, wild-type mice developed acute pancreatitis with high amylase and lipase serum levels. On the contrary, enzymatic levels in cerulein-treated ElaI-VMP1 mice were significantly lower compared with wild-type mice. Consistently, ElaI-VMP1 mouse pancreas showed remarkably less macroscopic evidence of acute pancreatitis compared with wild-type animals that showed marked edema and hemorrhage. Histological analyses displayed a high degree of necrosis as well as inflammation in wild-type pancreas with acute pancreatitis. In contrast, neither necrosis nor significant inflammation was seen in cerulein-treated ElaI-VMP1 mice [41,43]. Thus, results obtained in the transgenic animal model showed that zymophagy functions as a protective pathophysiological mechanism against pancreatitis-associated injury.

Upon CCK-R hyperstimulation, acinar cells from wild-type mice showed early cytoplasmic trypsinogen activation, which is a hallmark of pancreatitis pathophysiology. Surprisingly, in acinar cells from ElaI-VMP1 mice, CCK hyperstimulation caused almost no activation of trypsinogen. Microscopic examinations using BZiPAR (rhodamine 110 bis-[CBZ-L-isoleucyl-L-prolyl-L-arginine amide] dihydrochloride), a cell permeable substrate that becomes fluorescent after the cleavage by the protease revealed only few activated granules that highly colocalize with VMP1, showing that zymophagy selectively sequesters the activated zymogen granules. Zymogen activation is an enzymatic chain reaction where initial zymogen granule alterations trigger rapid spread of active trypsin within the acinar cell. We think that the

degradation of early-activated zymogen granules by zymophagy prevents this deleterious event. Interestingly, the inhibition of autophagic flow markedly increased trypsin activity within acinar cells in ElaI-VMP1 mouse pancreases under CCK-R hyperstimulation confirming that zymophagy specifically degrades those zymogen granules that are initially activated by acute pancreatitis [43]. This function is confirmed in the in vivo animal model of acute pancreatitis where the ability of the ElaI-VMP1 mouse developing zymophagy clearly prevents the increment of enzymatic markers of pancreatic damage and morphological changes characteristic of acute pancreatitis.

Analysis of autophagosomes containing zymogen granules magnetically immunopurified from the pancreas of ElaI-VMP1 mice treated with cerulein revealed that, apart from zymogen granules, isolated vesicles contained LC3-II and, notably, strong signals of p62. Moreover, GFP-ubiquitin-transfected acinar cells subjected to CCK-R hyperstimulation showed colocalization between activated granules and ubiquitin aggregates but do not show colocalization between unaffected or normal zymogen granules and ubiquitin, indicating that the ubiquitin system serves as a targeting signal for activated zymogen granules during zymophagy. Therefore, activated zymogen granules are directly or indirectly ubiquitinated for their recognition by autophagic membranes in which ubiquitin acts as a label for selective engulfment. Nevertheless, activated zymogen granules were ubiquitinated upon acute pancreatitis and the VMP1-mediated selective autophagic pathway sequestered these ubiquitinated granules [43]. p62 may function as a cargo receptor during zymophagy. These data demonstrate for the first time that ubiquitin modifications may possess an additional function in acinar cells by promoting the degradation of highly harmful activated zymogen granules [55].

Zymophagy prevents pancreatic acinar cell death induced by CCK-R hyperstimulation [43]. Autophagosome formation inhibition with 3-methyladenine as well as autophagy flux interruption with vinblastine significantly reduced acinar cell survival in a cell model of acute pancreatitis. Moreover, VMP1 downregulation (shVMP1) also significantly decreases acinar cell survival under CCK-R hyperstimulation, showing that VMP1 expression and autophagy is required to prevent acinar cell death in acute pancreatitis. Therefore, VMP1 expression is activated in acinar cells to mediate zymophagy as a protective cellular response against cell death [55].

Furthermore, VMP1 expression and zymophagy are present in human pancreas affected by acute pancreatitis [43,55]. VMP1 is not detectable in human normal pancreas tissue but its expression is activated in human pancreatitis pancreatic specimens and highly colocalized with LC3 in autophagosomes. Moreover, autophagosomes markedly colocalized with zymogen granules. Remarkably, the finding of large autolysosomes without trypsin signal in pancreas of human pancreatitis suggests that affected zymogen granules are eventually degraded by zymophagy during human pancreatitis.

## 3. Autophagy in cell fate

Though there was controversy about whether autophagy could be at the same time a survival cell response and a programmed cell death pathway, there is none that autophagy has the

power to change the fate of a cell. From an energetic homeostasis point of view, autophagy is a major cellular response against starvation condition. Nevertheless, we have to keep in mind that autophagy plays key roles also in other cellular stress conditions such as oxidative damage [57,58], damaged organelles elimination [59-61], depletion of toxic proteins aggregates [62], host response to microorganisms [61], etc. In those cases, autophagy is not just a way to keep energy and cellular material but a specific cellular stress response. Therefore, it is not difficult to imagine that autophagy could be observed in several cellular life-threatening situations and, then, its pro-survival or pro-death role becomes diffuse. Solid and ineligible is the fact that, independently, its situation-specific role, a determined autophagy process, has a profound impact in the cellular fate.

### 3.1. Autophagy as nutritional stress response

We must begin our analysis from the most basic and evolutionarily conserved autophagy duty as nutrient stress response. In the starvation context, autophagy plays the first explored, and may be more obvious, task of autophagy, that is, the energy cellular support during nutrient-limiting conditions. Though the bulk degradation and recycling of cytoplasmic portions seem to be a simple event in cellular life, it has major consequences in cell fate and organismal adaptation. As it could be imagined, a proper autophagic flux during a challenging cellular nutritional status might be determinant for the cell survival. Furthermore, the importance of starvation-induced autophagy for cell survival can have beneficial or detrimental consequences to tissue in context dependence. For instance, autophagy could give a life opportunity to cells under urgent energy requirement such as organ starvation, ischemia, hypoxia, etc., being beneficial for a determinate tissue. On the other hand, the same mechanism gives to pancreatic cancer cells enough adaptation to survive in a highly hipoperfunded environment of the pancreatic tumor [63]. This dual behavior could also be observed with AMPK (5' AMP-activated protein kinase) which is one of the master regulators of the cellular energetic homeostasis and a direct autophagy trigger through ULK1 phosphorylation [64]. Similar to what is observed with autophagy, AMPK could be a tumor suppressor, stopping all anabolic process and activating all the catabolic ones including autophagy (probably contributing to the oncogene-induced senescence – see below) but, in other circumstance, it gives tumor high resistance to stress [65].

### 3.2. Autophagy and cell death

Connections between autophagy and disease have attracted an increasing amount of attention. By morphological studies, autophagy has been linked to a variety of pathological processes and autophagy was associated with cell death. Taking cell death as one major topic in cell fate, there are a large amount of data concerning the complex relationship between autophagy and apoptosis [65,66]. However, this relationship is far from being fully understood and, in many cases, it seems to be context-dependent. This autophagy–apoptosis relationship could be observed from the beginning since they share several inducing factors such as ROS [57], increased levels of cytosolic calcium concentrations [67], oncogenes, and p53 [68], among others (Figure 4A). Among those, the BH3-only proteins have a prominent role. These are pro-

apoptotic and different from the Bcl-2 family proteins which contain only one BH3 (Bcl-2 homology 3) domain [69]. BECN1 is a fundamental protein in autophagic mechanism (as mentioned above). This protein was one of the first described mammalian autophagy-related proteins [70]. BECN1 interacts with proteins of Bcl-2 family (Bcl-2, Bcl-XL and Mcl-1) [71]. Through interaction with the BECN1 BH3 domain, these proteins are able to inhibit BECN1-mediated autophagy [72, 73]. What is more, this interaction makes BECN1 not only a highly relevant autophagy actor but it is indeed also a haploinsufficient tumor suppressor [74]. On the other hand, BH3-only proteins are capable of disruption of Bcl-2-BECN1 interaction inducing apoptosis by Bcl-2 blockade and autophagy by releasing BECN1 [75].

Experimentally, impairment of autophagy in starving cells is able to induce apoptosis or at least a kind of cell death. This autophagy shortage impedes cell proper management of a metabolic stress or, in some cases, such as neurons, clearance of toxic cellular metabolic products. Moreover, in some way, this effect seems to be in both sides since inhibition of apoptosis could also be a potent trigger of the autophagy process. Transgenic depletion of pro-apoptotic molecules such as Bax or Bak or the use in vitro of the pan-caspase inhibitor Z-VAD-FMK were able to strongly induce autophagy and a cell death which is inhibited by autophagy inhibitors [76,77]. This seems to be the rule in most cases, a highly intricate autophagy-apotosis cross talk, where, despite their respective main duties, they act by inhibiting each other (Figure 4A). For instance, in one way, autophagy could reduce the cytosolic concentrations of pro-apoptotic proteins [78]. In the other way, activated caspases are able to cleave some autophagic proteins in which the fragmented products indeed acquired now pro-apoptotic properties [79-83]. Such is the case of ATG5, BECN1, and ATG4D [79-83].

Beyond the central role of ATG5 in autophagy machinery, this protein, in some situations, is able to induce apoptosis by two different ways. As mentioned above, a calpain-mediated cleaved ATG5 translocated to mitochondria, favoring its depolarization and triggering of apoptosis [84]. Moreover, ATG5 can also associate to FADD (Fas-associated death domain) enhancing apoptosis cell death [85].

The autophagic cell death is a programmed alternative to other sorts of cell demise. All of the comments above about the relationship of autophagy with cell death do not imply that autophagy could be by itself a cell death mechanism. Many researchers have suggested a number of times the existence of a really programmed autophagic cell death. Nevertheless, since autophagy is a cell survival pathway, there was concern about whether autophagy was a last cellular attempt to avoid apoptosis or if autophagy-related cell death only occurs in in vitro settings. These controversies were clarified with study of the degeneration of salivary glands during the D. melanogaster embryogenesis [86]. These glands suffered a cell death that is independent of caspases and completely dependent on autophagy [87].

All this intricate cross talk among apoptosis, autophagy, and autophagic cell death makes one rethink the definition of cell death types. Hence, the 2015 Nomenclature Committee on Cell Death made a switch from morphological- to biochemical-based definitions of cell death types [87]. They stated that autophagic cell death will be defined as processes of cell death that were

prevented by the use of pharmacological or genetic tools targeting at least two different components of autophagy machinery [87].

Beyond the molecular mechanism, autophagy is able to modulate the cell death response and, hence, cell fate indirectly. Mitophagy, the most studied form of selective autophagy, is aimed to eliminate damaged mitochondria contributing to its renewing and ROS homeostasis [88]. As mitochondrial impairment is one of most important apoptosis triggering events, elimination of damaged mitochondria could avoid the intrinsic apoptosis program (Figure 4B). Damaged mitochondria and subsequent decrease in the inner mitochondrial transmembrane potential ($\Delta\ \Psi$) led to leakage of several harmful substances such as ROS and intrinsic apoptotic-pathway-triggered leakage of others such as cytochrome c [88]. In response, damaged mitochondria may be fragmented and ubiquitinated by a PINK-Parkin-dependent mechanism [89]. Then, these damaged organelles are recognized and selectively eliminated by autophagy in order to allow the cell survival [64,83].

It is surprising that autophagy can also interfere with such an uncontrolled process as necrosis. This example was demonstrated to occur during acute pancreatitis with zymophagy [55]. In the cellular basis of this disease, there was a premature intracytoplasmic activation of digestive enzymes [41]. This last activity pushes pancreatic acinar cell to an inevitable necrosis and the catastrophe of the tissue in a sort of chain cascade [41]. Zymophagy, as selective type of autophagy, eliminates the granules where the dangerous zymogens were activated by a ubiquitin recognition mechanism [55]. This protective mechanism avoids the enzymatic autodegradation and subsequent necrosis of the acinar cell [55] (Figure 4B). This event, in fact, has consequences beyond cellular biology since it reduces gland inflammation and contributes to autolimitation of the disease.

### 3.3. Autophagy and cellular senescence

There exists another unexpected process related to cell fate which is influenced by autophagy: the cellular senescence [90]. This term is used to describe an irreversible (which differentiates it from quiescence) deep arrested status of the cell cycle. The senescence is a cellular alternative mechanism to apoptosis in response to certain stressors including oncogene overexpression. Then, the so-called oncogene-induced senescence is a potent tumor suppressor mechanism against cellular transformation [91-94] and it is the first option that cells have against oncogene activation. Autophagy participated in the transition phase of senescence establishment being part of the TOR-autophagy spatial coupling compartment (TASCC). The TASCC is a cytoplasmic sub-compartmentalization where mTOR is closely associated with lysosomes/autolysosomes fuelled by a constant autophagy flow outside of this area [95]. This activity resulted in an efficient synthesis-degradation coupling that seems to be crucial for senescence. Inhibition of autophagy impairs the senescent progression and it seems to be necessary to reach an intermediate state before the final setting of senescent phenotype. Finally, it was suggested that autophagy during senescence is triggered by the isoform ULK3 instead of ULK1 or ULK2; therefore, it is tempted to hypothethize that a specific type of autophagy may be related to senescence (Figure 4B).

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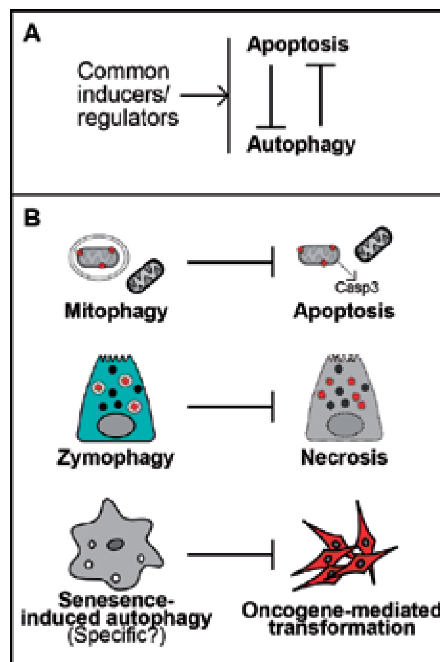


**Figure 4.** The implication of autophagy in cell fate. A) There is a deep crosstalk between autophagy and apoptosis. They share several common inducers and posses regulatory properties on each other. B) Autophagy is able to modify cell fate and some examples are depicted. Mitophagy eliminates the damaged mitochondria avoiding the apoptosis triggered by intrinsic pathway (upper panel). By the specific elimination of activated zymogen granules, zymophagy prevents the acinar cell damage and the necrosis in pancreas tissue (middle panel). Autophagy participates in the oncogene-induced senescence, a process capable of repress the oncogenic transformation (lower panel).

## 4. Autophagy in pancreatic diseases

Pathological processes such as pancreatitis and diabetes mellitus as well as cancer cell transformation and also cancer chemotherapy activate autophagy in human tissues and human tumor cells. While human normal pancreatic acinar cells do not have detectable autophagy levels, pancreatic diseases activate autophagy, confirming the relevant role of autophagy in human pancreatic disease. In addition, selective autophagy of pancreatic zymogen granules, zymophagy, has been discovered and characterized as a cell-protective process activated by the disease.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human malignancies with 2-3% five-year survival rate. It remains a devastating and poorly understood malignancy. Its poor prognosis has been attributed to the inability to make a diagnosis while the tumor is still resectable and a propensity toward early vascular dissemination and spread to regional lymph nodes. Up to 60% of patients have advanced pancreatic cancer at the time of diagnosis and their median survival time is a dismal 36 months. This is due to both the aggressive nature

of the disease, the lack of specific symptoms and early detection tools, and its relatively refractory response to traditional cytotoxic agents and radiotherapy. Moreover, pancreatic cancer cells become more malignant or survive with an extremely poor blood supply. So far, little and contradictory data are available regarding the activity of autophagy and its regulation in pancreatic cancer cells. Experimental evidence pointed at autophagy as a pancreatic cancer cell mechanism to survive under adverse environmental conditions or as a defective programmed cell death mechanism that favors pancreatic cancer cell resistance to treatment.

### 4.1. Autophagy in cancer cell

Both downregulated and excessive autophagy have been implicated in the pathogenesis of diverse diseases such as certain type of neuronal degeneration, diabetes and its complications, and cancer [96]. Autophagy has also been implicated in cell death called autophagic or type II programmed cell death, which was originally described on the basis of morphological studies detecting autophagic vesicles during tissue involution [97].

Cancer cells in general tend to undergo less autophagy than their normal counterparts at least for some tumors [98,99]. The BECN1 autophagy gene is monoallelically deleted in 40-74% of cases of human sporadic breast, ovarian, and prostate cancer [99]. Heterozygous disruption of BECN1 increases the frequency of spontaneous malignancies and accelerates the development of virus-induced premalignant lesions [99] suggesting that defective regulation of autophagy promotes tumorigenesis. It has been proposed that autophagy suppresses carcinogenesis by a cell-autonomous mechanism involving protection of genome integrity and stability, and a nonautonomous mechanism involving suppression of inflammation and necrosis. On the other hand, autophagy may support the survival of rapidly growing cancer cells that have outgrown their vascular supply and are exposed to an inadequate oxygen supply or metabolic stress. By contrast, excessive levels of autophagy promote cell death [100]. Accordingly, it has been proposed that autophagy plays an important role both in tumor progression and in promotion of cancer cell death [101], although themolecular mechanisms responsible for this dual action of autophagy in cancer have not been elucidated.

It has been suggested that autophagy may be a cancer cell survival response to tumor-associated hypoxia. Tumor hypoxia has been used as a marker of poor prognosis [102]; however, how cancer cells become more malignant or survive with an extremely poor blood supply is poorly understood. When cancer cells are exposed to hypoxia, anaerobic glycolysis increases and provides energy for cell survival but as the glucose supply is also insufficient because of the poor blood supply, there must be an alternative metabolic pathway that provides energy when both oxygen and glucose are depleted [103,104]. Hypoxia in pancreatic cancer has been reported to increase its malignant potential [102]. Proliferating cancer cells require more nutrients than surrounding noncancerous cells do, though nutrition is supplied via functionally and structurally immature neovessels. Because autophagy-specific genes promote the survival of normal cells during nutrient starvation in all eukaryotic organisms, autophagy may react to the cancer microenvironment to favor the survival of rapidly growing cancer cells. LC3 expression in surgically resected pancreatic cancer tissue showed activated autophagy in the peripheral area, which included the invasive border, and concomitantly

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shows enhanced expression of carbonic anhydrase [105]. This observation suggests that autophagy may promote cell viability in hypovascularized cancer tissue.

It has also been proposed that autophagy is a cancer cell survival response to tumor-associated inflammation [106]. Cancer-associated inflammation results in promotion of carcinogenesis and resistance to therapy. Several phenotypic alterations observed in cancer cells are a result of inflammatory signals found within the tumor microenvironment [106]. The receptor for advanced glycation end products (RAGE) is an induced inflammatory receptor constitutively expressed on many murine and human epithelial tumor cell lines [107,108] and the highest levels of RAGE expression were observed in murine and human pancreatic adenocarcinoma tumors. Genotoxic and/or metabolic stress lead to modest but reproducible increases in overall expression of RAGE on epithelial cell lines. RAGE expression correlates directly with the ability of both murine and human pancreatic tumor cell lines to survive cytotoxic insult. Targeted knockdown of RAGE significantly increased cell death, whereas forced overexpression promotes survival. Recently, it was reported that the enhanced sensitivity to cell death in the setting of RAGE knockdown is associated with increased apoptosis and decreased autophagy. In contrast, overexpression of RAGE is associated with enhanced autophagy, diminished apoptosis, and enhanced cancer cell viability. Knockdown of RAGE enhances mTOR phosphorylation in response to chemotherapy, thus preventing induction of a survival response. Inhibition of autophagy by means of silencing BECN1 expression in pancreatic cancer cells enhanced apoptosis and cell death [109]. These observations suggest that RAGE expression in cancer cells has a role in tumor cell response to environmental stress through the enhancement of autophagy. However, increased sensitivity to chemotherapeutic agents in RAGE-knockdown pancreatic cancer cells is dependent on ATG5 expression but independent of BECN1 expression [109]. These last findings suggested that the role of autophagy in the resistance to microenvironment insult or in the sensitivity to chemotherapeutic agent is the result of complex molecular pathways in the tumor cell.

On the other hand, repression of autophagy has been suggested as a cancer cell response to prolonged hypoxic conditions. Pancreatic cancer cell response to prolonged hypoxia may consist of inhibition of autophagic cell death. The short isoform of single-minded 2 (SIM2s) is a member of the basic helix-loop-helix family of transcriptional regulators [110] and is upregulated in pancreatic cancer. Microarray studies identified the pro-cell death gene BNIP3 as a target of SIM2s repression. Prolonged hypoxia induces cell death via an autophagic pathway involving the hypoxia-inducible factor 1 (HIF1)-mediated upregulation of BNIP3 [30,111]. Deregulation of both SIM2s and BNIP3 were associated with poor prognostic outcomes [112]. Decreased BNIP3 levels and poor prognosis clearly correlate with elevated SIM2s expression in pancreatic cancer. The loss of BNIP3, either by hypermethylation or by transcriptional repression, was correlated with inhibition of cell death [113, 114], whereas upregulation of BNIP3 sensitized pancreatic carcinoma cells to hypoxia-induced cell death [115]. SIM2s expression, concomitant with its repression of BNIP3, enhanced tumor cell survival under prolonged hypoxic conditions. Recent data linked increased SIM2s expression with enhanced cell survival during hypoxia-stress concomitantly with BNIP3 repression and the attenuation of hypoxia-induced autophagic processes.

Thus, inhibition of autophagic cell death by BNIP3 repression enhances tumor cell survival under prolonged hypoxic conditions [115].

Decreased autophagy in some cancer cells has been related to malignant stages of the disease. Cancer cells in general tend to undergo less autophagy than their normal counterparts supporting the contention that defective autophagic cell death plays a role in tumor progression. Studies of carcinogen-induced pancreatic cancer in animal models have shown that pancreatic adenocarcinoma cells have lower autophagic capacity than premalignant cells [116]. The WIPI protein family, which includes ATG18, the WIPI-1 homolog in S. cerevisiae, was genetically identified as a gene contributing to autophagy [116]. Human WIPI-1a is a member of a highly conserved WD-repeats protein family. hWIPI-1 is linked to starvation-induced autophagy in the mammalian system. Amino acid deprivation triggered an accumulation of endogenous hWIPI-1 protein to large vesicular and cup-shaped structures where it colocalizes with LC3. Starvation-induced hWIPI-1 formation was blocked by wortmannin, a principal inhibitor of PI-3 kinase-induced autophagosome formation [117]. Interestingly, WIPI proteins are linked pathologically to cellular transformation because all human WIPI genes are reportedly expressed aberrantly in a variety of matched human cancer samples. Strikingly, hWIPI-2 and hWIPI-4 mRNA expression is substantially decreased in 70% of matched kidney (10 patients) and 100% of pancreatic (seven patients) tumor samples. The majority of these samples were derived from advanced-stage tumors such as pancreatic adenocarcinomas stages I–IV. Hence, cancer-associated downregulation of hWIPI-2 and hWIPI-4 supports the possibility that decreased autophagic activity is necessary for the malignant stages of pancreatic cancer.

## 5. Perspectives

There is ample evidence supporting an active role for autophagy in cell physiology and disease. During the last decade, autophagy has turned from a morphological finding to a cellular process involving a membrane transport system and complex molecular machinery. Moreover, since the discovery of ATG genes, there have been many studies on the physiological and pathological roles of autophagy in a variety of autophagy knockout models. However, direct evidence of the connections between ATG gene dysfunction and human diseases has emerged only recently [56]. Here we have overviewed the physiological bases and molecular mechanisms of the autophagic process. We have introduced the reader to a novel autophagy-related transmembrane protein – VMP1 – whose expression triggers autophagy and its role in the cell response to disease. Elucidation of the specific extracellular and intracellular conditions that stimulate autophagy and the linkage of these conditions to either cell survival or cell injury and death in different cell types and during different pathological processes is a rapidly evolving and fruitful field of research. The development of therapies to take advantage of the potential cytoprotective effect of autophagy in several pathologies such as cancer or neurodegenerative diseases is a potentially promising avenue of investigation.

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Chapter 2

# Interconnected Regulation of Apoptosis and WIPI-Mediated Autophagy

Katharina Sporbeck, Fenja Odendall and Tassula Proikas-Cezanne

Additional information is available at the end of the chapter

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**Abstract**

Autophagy is a genetic program that secures the survival of eukaryotic cells to compensate for periods of starvation and cellular stress. Apoptosis, in contrast, is a genetically defined program leading to cell death. Both pathways are interconnected through conserved co-regulatory signaling pathways that are context-dependent. Proper co-regulation of autophagy and apoptosis, whereby autophagy exerts an antiapoptotic function and apoptosis inhibits autophagic survival strategies, critically secures the survival of healthy cells and counteracts genomic instability in eukaryotic organisms. Cancer cells often become resistant to apoptosis and addicted to autophagy, making this scenario highly relevant to define novel therapeutic strategies. The co-regulatory crosstalk between apoptosis and autophagy converges on the production of phosphatidylinositol 3-phosphate (PI3P) essential for the onset of autophagy. WD-repeat protein interacting with phosphoinositides (WIPI) members function as essential PI3P effectors in autophagy and fulfill an important role in health and disease. Here, we summarize details on the regulation of WIPI-mediated autophagy in the context of co-regulatory signals for both apoptosis and autophagy.

**Keywords:** WIPI, PI3P, autophagy, autophagosomal cell death, p53

## 1. Introduction

The process of autophagy was conceptualized in 1963 by Christian de Duve, who coined the term based on the Greek words *auto* for self and *phagy* for eat, hence self-eating. de Duve suggested that intracellular vesicles, derived from the endoplasmic reticulum, harbor cytoplasmic material for lysosomal degradation. This novel concept was followed up by morphological studies combined with biochemical analysis, providing compelling evidence that autophagy represents an evolutionarily conserved catabolic machinery in eukaryotic cells for

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the degradation of proteins, lipids, and organelles, producing monomers and energy in periods of starvation. The discovery of autophagy-related (ATG) genes that initiate and execute autophagy laid the foundation to dissect the process of autophagy in molecular detail and, moreover, provided the basis to realize that autophagy is intrinsic to cellular survival [1].

The term apoptosis was introduced by John Kerry, Andrew Wyllie, and Alastair Currie in 1972, based on the Greek words *apo* for off and *ptosis* for fall, hence falling off, referring to the leaves falling off the tree. The term was introduced to define a discrete cell death morphology including cell shrinkage, fragmentation, and blebbing, dissimilar to the process of necrosis characterized by cell swelling and lysis, subsequently provoking an inflammatory response. Studies in *Caenorhabditis elegans* (*C. elegans*) laid the foundation to discover the molecular basis of apoptotic cell death, ultimately leading to the current general knowledge that apoptosis is a genetic program of cell death [2].

Although both evolutionarily conserved genetic programs autophagy and apoptosis result in opposing consequences, cellular survival or death, respectively, both are intrinsic to the development, differentiation, and health of eukaryotic organisms, critically secure genomic stability, and fight the onset of age-related human diseases such as cancer [3, 4]. Deduced from this, autophagy and apoptosis should, despite representing distinct genetic programs, be interconnected by conserved context-dependent control mechanisms. In fact, this hypothesis has attracted an enormous amount of interest over the last decade or more, resulting in a great quantity of publications available on this topic. Generally accepted now is the consideration that: i) autophagy inhibits apoptosis and ii) apoptosis inhibits autophagy [5]. However, under certain circumstances autophagy, in particular forms of noncanonical autophagy, contributes to cell death [6]. Moreover, it has been recognized that cell death can also occur by excessive autophagy, referred to as autophagic cell death (ACD). In this context, it is considered that autophagy specifically restricts ACD but that superabundant autophagic degradation mediates ACD [5].

In this chapter, we focus on canonical WD repeat protein interacting with phosphoinositides (WIPI)-mediated autophagy [7] and its regulation through common regulatory factors for both autophagy and apoptosis.

## 2. The process of WIPI-mediated autophagy

The regulatory relationship between autophagy and apoptosis converges in controlling phosphatidylinositol 3-phosphate (PI3P) production, which initiates autophagosome formation through the PI3P-effector function of WIPI proteins at the onset of autophagy (Figure 1). PI3P production is initiated to induce autophagy upon nutrient or energy deprivation [1]. Low energy levels in the cell activate the AMP-activated kinase (AMPK), which phosphorylates and thereby activates the serine/threonine-specific protein kinase ULK1 (UNC51-like kinase 1) functioning in a principal regulatory complex together with FIP200 (focal adhesion kinase family interacting protein of 200 kD) [8]. AMPK is further controlled by the $Ca^{2+}$/calmodulin-dependent protein kinase β (CaMKKβ) [9] and liver kinase B1 (LKB1) [10]. However, amino acid deprivation has also been shown to initiate WIPI-mediated autophagy in the absence of AMPK [11].

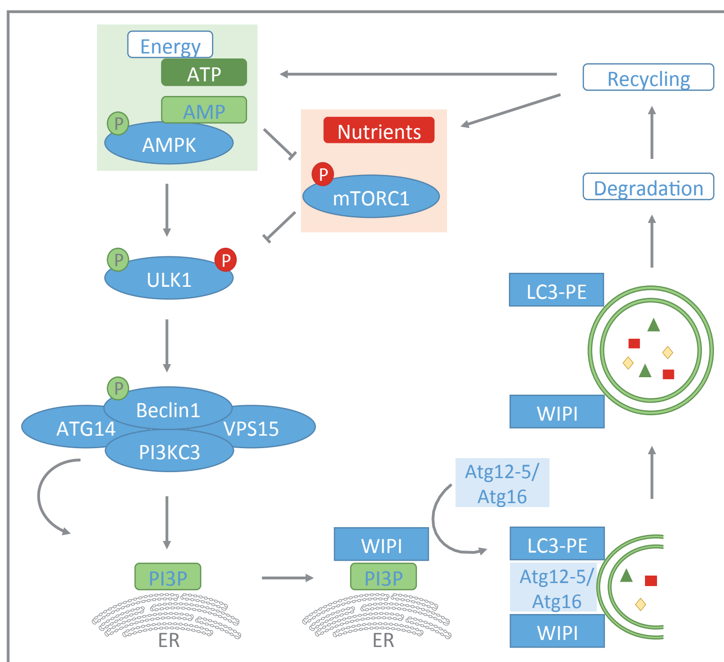


**Figure 1.** A current working model of WIPI-mediated autophagy

Active ULK1, in complex with FIP200, was shown to phosphorylate Beclin 1, a regulatory factor that in turn forms a complex with the phosphatidylinositol 3-kinase class 3 (PI3KC3), vacuolar protein sorting-associated protein 15 (VPS15) and autophagy-related protein 14 (ATG14) [12]. This core PI3KC3 complex localizes through ATG14 to the endoplasmic reticulum and produces PI3P essential for the formation of initial autophagosomal membranes referred to as phagophore or isolation membrane [13]. The PI3P signal is decoded by WIPI proteins, members of the PROPPIN (beta-propellers that bind phosphoinositides) family with the four human members WIPI1 to WIPI4 considered to function as essential PI3P effectors at the nascent autophagosome (Figure 1) [7, 14].

The identification of human WIPI members was based on initial screening of a human liver cDNA library for novel inhibitors of p53 and it was shown that WIPI genes are ubiquitously expressed in normal human tissue but aberrantly in a variety of human cancer types [14]. By structural homology modeling, it was found that WIPI proteins fold into seven-bladed beta-propellers with an open “Velcro” arrangement harboring a phosphoinositide-binding site specific for the PROPPIN family [7, 14]. Initial phylogenetic analysis revealed that the PROPPIN family consists of two paralogous groups harboring WIPI1/WIPI2 and WIPI3/4 in each of the groups, respectively [14].

WIPI1 is the founding member of the human PROPPIN group [7, 14] currently with the two recognized splice variants WIPI1$\alpha$ [7] and WIPI1$\beta$ (= WIPI49) [15] WIPI1 plays an important role in autophagy [16] due to the specific binding to PI3P [17]. Upon autophagy induction, WIPI1 localizes to the endoplasmic reticulum, and is then found on the phagophore, and the

autophagosome (Figure 1) [18]. Due to this specific localization, quantitative assessment of PI3P-binding dependent association of WIPI1 with autophagosomal membranes has been established to monitor autophagy in mammalian cells using automated high-throughput imaging [19].

WIPI2 also binds to the phagophore and to autophagosomes due to its specific binding to PI3P [7,18,20]. By comparing four of the many existing WIPI2 splice variants [14], it became apparent that only WIPI2B and WIPI2D, both expressed upon exon skipping, are involved in autophagy but that full-length WIPI2A and also WIPI2C do not seem to respond with an increase in autophagosomal membrane localization upon amino acid starvation [16]. Importantly, WIPI2B was recently shown to specifically recruit components of the autophagy-specific ubiquitin-like conjugation system to the phagophore [21]. Hereby, WIPI2-dependent recruitment of the ATG12-5/ATG16 complex permits the conjugation of LC3 to phosphatidylethanolamine, a process often referred to as LC3 lipidation (Figure 1) [21]. PE-conjugated LC3 is subsequently engaged in phagophore expansion and specific cargo recruitment. LC3 as well as WIPI1 and WIPI2 become membrane proteins of autophagosomes (Figure 1).

So far, PI3P binding as well as autophagosomal membrane localization has not been demonstrated for WIPI3. However, WIPI4 is considered to also bind to the phagophore but downstream of WIPI1 and WIPI2 [7, 22]. Due to the identification of novel *de novo* mutations in WIPI4, loss of WIPI4 function has been linked to SENDA (static encephalopathy of childhood), a sporadic form of NBIA (neurodegeneration with brain iron accumulation) [23]. Based on this finding and the important roles of WIPI proteins in executing autophagy in general, it is predicted that WIPI malfunctions may be intrinsic to a great variety of human pathologies with irregular autophagy [7].

An important evolutionarily conserved inhibitor of autophagy is mTOR (mammalian target of rapamycin). mTOR is a serine-threonine-specific protein kinase and component of two multiprotein complexes, mTORC1 (mTOR complex 1) and mTORC2 (mTOR complex 2) [24]. mTORC1 consists of RAPTOR (regulatory-associated protein of mTOR), PRAS (40kDa Pro-rich Akt substrate), mLST8 (mammalian lethal with SEC13 protein 8), and DEPTOR (DEP domain containing mTOR interacting protein). mTORC1 represents a nutrient sensor in eukaryotic cells and, in its activated form, mTORC1 localizes at the lysosomal surface and receives free amino acids released from the lysosome [24]. mTORC1 can also be activated by a great variety of signaling cascades, most prominently by the insulin/IGF (insulin-like growth factor) receptor pathway via AKT [24]. Importantly, amino acid availability activates mTORC1 and, in consequence, inhibits autophagy. The inhibition of autophagy by mTORC1 is mediated through ULK1 phosphorylation (Figure 1). In this situation, mTORC1 also phosphorylates S6K1 (ribosomal S6-kinase 1) and 4E-BP (eIF4E binding protein), which enhances autophagy-opposing effects, mRNA translation, and protein synthesis [24].

## 3. Signals that regulate both apoptosis and WIPI-mediated autophagy

Autophagy and apoptosis are co-regulated by a complex signaling crosstalk, generally leading to the inhibition of apoptosis when autophagy is active and the inhibition of autophagy to permit apoptosis (Figure 2).

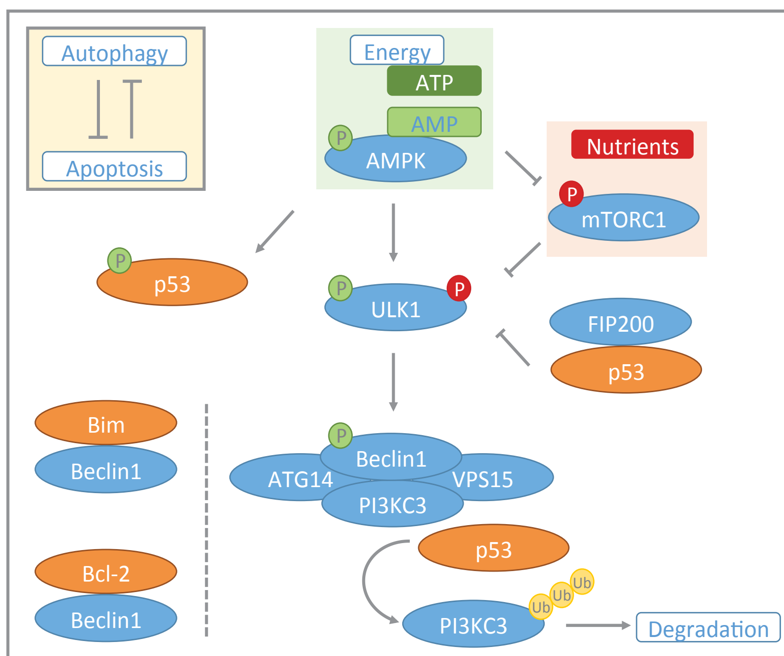


**Figure 2.** Regulation of autophagy by factors that also regulate apoptosis

Autophagy negatively regulates apoptosis by the specific degradation of apoptotic components including active caspase-8 [25] and the proto-oncogenic c-src (cellular src; src for sarcoma) tyrosine kinase that can initiate apoptosis by activating pro-caspase-9 [26]. Thereby, active c-src is recruited to the phagophore through CBL (casitas B-lineage lymphoma)-mediated binding to LC3 [26]. Subsequently, c-src is degraded through autophagy.

Another apoptosis-preventing result of autophagic activity is the specific removal of damaged mitochondria, termed mitophagy [27]. Depolarization of the mitochondrial membrane indicates a loss of function in the respiratory chain and leads to an accumulation of PINK1 (PTEN-induced putative kinase 1) on the outer mitochondrial membrane. PINK1 recruits and activates the E3 ubiquitin ligase PARKIN that ubiquitinates VDAC1 (Voltage-dependent anion-selective channel protein 1) and MFN1/2 (Mitofusin-1/2). Ubiquitinated VDAC1 and MFN1/2 are recognized by p62 that connects the damaged organelle via LC3 binding to the phagophore [27]. Hence, intrinsic activation of apoptosis due to defective mitochondria is counteracted by autophagy.

A crucial co-regulator of both autophagy and apoptosis is AMPK, which initiates autophagy through ULK1 phosphorylation (Figure 2). Interestingly, active AMPK phosphorylates p53 at serine 15 upon glucose starvation, which activates the transcriptional transactivator activity of p53 to promote an AMPK-dependent cell-cycle arrest at the G1/S boundary [28]. As a consequence, reentering the cell cycle is restricted to glucose availability, securing that dividing cells have enough nutrients available for energy and macromolecule production [28].

On the other hand, p53 inhibits autophagy in several distinct ways (Figure 2). In exhibiting its nonnuclear function, cytoplasmic p53 competes with ULK1 for binding to FIP200 under certain circumstances [29]. As the ULK1/FIP200 association is required for the activation of PI3KC3 and subsequent PI3P production followed by WIPI-mediated autophagy (Figure 1), cytoplasmic p53 inhibits autophagy. This scenario represents an interesting connection to the induction of apoptosis, which can be initiated through the association of cytoplasmic p53 to mitochondria [30].

In general, the site-specific transcriptional transactivator factor activity of p53 in the nucleus is induced upon DNA damage and p53 target genes subsequently permit DNA repair or apoptosis to secure genomic stability. Among the p53 target genes, FBXL20 (F-box and leucine-rich repeat protein 20), known to mediate ubiquitination and proteasome-mediated degradation, is expressed and targets PI3KC3 [31]. Hence, activation of the nuclear function of p53 can also lead to the inhibition of autophagy through p53-promoted PI3KC3 degradation (Figure 2).

Further, common factors regulating autophagy and apoptosis include Bcl-2 (B-cell lymphoma 2) with antiapoptotic function and Bim (Bcl-2-interacting mediator of cell death), a BH3 (Bcl-2 homology domain 2)-only protein with pro-apoptotic function (Figure 2). Bim is bound to the dynein motor protein DYNLL1/LC8 (dynein light chain 1) at microtubules. In nutrient-rich conditions, Bim binds to Beclin 1, preventing Beclin 1 binding to complex with PI3KC3 and initiate WIPI-mediated autophagy. Bim, therefore, inhibits autophagy through Beclin 1 mislocalization to the dynein motor protein [32]. In starvation conditions, Bim releases Beclin 1 and allows Beclin 1/PI3KC3 association and subsequently the induction of autophagy [32].

Bcl-2, a well-known interacting partner of Beclin 1, also prevents Beclin 1 from binding to PI3KC3. The Bcl-2/Beclin 1 association is inhibited upon starvation, leading to JNK (c-Jun N-terminal kinase)-mediated Bcl-2 phosphorylation at multiple sites subsequently releasing Beclin 1 and permitting autophagy activation (Figure 2) [33].

The complex relationship between apoptosis and autophagy regulation is further highlighted by the notion that autophagy-related proteins fulfill pro-apoptotic functions under certain circumstances. During apoptosis, active caspases target AMBRA1, a component of the PI3KC3 complex, for degradation and hence prevent WIPI-mediated autophagy upon the apoptotic point-of-no-return route to cell death [34]. Further, it was demonstrated that calpain 1 and calpain 2, classified as cysteine proteases [35], cleave ATG5 at threonine 193 generating an 24 kDa N-terminal fragment of ATG5 [36]. This ATG5 fragment subsequently translocates to the mitochondrial membrane and initiates cytochrome c release, ultimately promoting the cleavage of pro-caspase-3 into its active form. The effect of fragmented ATG5 is inhibited by high Bcl-2 levels [36]. Moreover, ATG12, the conjugation partner of ATG5, was shown to interact with Bcl-2 via an amino acid sequence in ATG12 resembling a BH3 domain generally known to bind Bcl-2. By binding to Bcl-2, ATG12 inhibits the antiapoptotic function of Bcl-2, leading to an increase of apoptotic cell death [37].

To provide a final example for the complex regulatory crosstalk between apoptosis and WIPI-mediated autophagy, the function of DAPK (death-associated protein kinase 1), a $Ca^{2+}$/

calmodulin-sensitive serine/threonine kinase involved in the induction of apoptosis [38], is highlighted in the following section. DAPK phosphorylates Beclin 1 or PKD (protein kinase D) under certain conditions. When reactive oxygen species activate DAPK, DAPK phosphorylates PKD, resulting in the phosphorylation of PI3KC3 by PDK. In turn, PI3KC3 produces PI3P and initiates autophagy [39]. As mentioned, DAPK can also phosphorylate Beclin 1, which occurs at threonine 119 in the BH3-like domain of Beclin 1 responsible for binding to Bcl-2. As Bcl-2-bound Beclin 1 inhibits autophagy, threonine 119 phosphorylation releases Beclin 1 from Bcl2 and enables Beclin1 to bind to PI3KC3 and to stimulate autophagy [40].

## 4. Outlook

In general, apoptosis and autophagy represent mutually exclusive genetic programs co-regulated by key factors with opposing roles in both pathways. This crosstalk contributes to the survival of healthy cells and secures genomic stability more than one of the pathways alone. Interestingly, key regulatory factors for both apoptosis and autophagy converge on the regulation of PI3P production. WIPI proteins function as essential PI3P effectors at the onset of autophagy; hence, PI3P-dependent localization of WIPI proteins at autophagosomal membranes should reflect both induction of autophagy and inhibition of apoptosis.

The genetic program of apoptosis is executed when the point-of-no-return is reached. In contrast, autophagy was shown to be initiated by the same stimuli that are additionally capable of blocking final autophagic cargo destruction in the lysosomal compartment, or even superstimulate autophagic degradation leading to autophagosomal cell death. Hence, the autophagic pathway not only represents a new rational therapeutic target mechanism for future treatment of human pathologies but also a target mechanism with the benefit to be modulated in many different ways according to the context-dependent needs.

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Chapter 3

# Autophagy Regulation of the Tumor Immunity – An Old Machinery for a New Function

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Additional information is available at the end of the chapter

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**Abstract**

Cancer was initially thought to be just a disease of cells with deregulated gene expression. It may be more accurate to consider cancer as a disease of the microenvironment. Despite the remarkable and fairly rapid progress over the past two decades regarding the role of the microenvironment in cancer biology and treatment, our understanding of its actual contribution to cancer resistance is still poor and fragmented. Nevertheless, the microenvironment is now considered to be of critical importance during the initiation and progression of carcinogenesis since it is involved in shaping and remodeling stroma reactivity and in reprogramming phenotypic and functional plasticity. Therefore, the tumor microenvironment represents an important hallmark of cancer, and the challenge now is to better understand how the tumor microenvironment participates in the emergence of immune-resistant tumor cell variants, which appears to be the greatest impediment to successful immunotherapy. In this context, autophagy has recently emerged as a new player in regulating the antitumor immune response under hostile tumor microenvironment. In this review, we will summarize recent data describing how autophagy activation under hypoxic stress impairs the antitumor immune response. In addition, we will discuss how tumor manages to hide from the immune attack and either mounts a "counterattack" or develops resistance to immune cells. In particular, we will focus on the effect of hypoxia-induced autophagy in allowing tumor cells to outmaneuver an effective immune response and escape from immunosurveillance. It is our belief that autophagy may represent a conceptual realm for new immunotherapeutic strategies aiming to block immune escape and therefore providing rational approach to future tumor immunotherapy design.

**Keywords:** Autophagy, hypoxia, tumor immunity, immunotherapy, immune surveillance

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# 1. Introduction

In addition to malignant cells, tumors contain cells of the immune system, the tumor vasculature, and lymphatics as well as fibroblasts, pericytes, and adipocytes. Cells of the immune system can identify and destroy tumor cells in a process termed cancer immunosurveillance.

Several types of immune cells are involved in tumor immune surveillance. Briefly, key cells of the adaptive immune system identifying cancer cells are cytotoxic T lymphocytes (CTL), which are able to recognize tumor antigens via the T-cell receptor (TCR) [1]. Some of these antigens are expressed exclusively by tumors and thus are called tumor-specific antigens [1]. Natural killer (NK) cells of the innate immune system also play an important role in tumor immune surveillance [1] by mechanisms called "missing-self" and "induced-self" recognitions [2]. In addition to CTL and NK cells, macrophages and neutrophil granulocytes are also involved in antitumor immunity [3]. Macrophages are antigen presenting cells (APCs) that display tumor antigens and stimulate other immune cells such as CTL, NK cells, and other APCs [4]. While the molecular mechanism by which CTL and NK cells recognize their target tumor cells is fundamentally different, both immune cells kill their target following the establishment of immunological synapse (IS) [5]. The formation of IS requires cell polarization and extensive remodeling of the actin cytoskeleton at various stages [6]. It is now well established that CTL and NK cells recognize and kill target cells by two major pathways: either through the release of cytotoxic granules containing perforin and granzymes to the cytosol of target cells [7] or through tumor necrosis factor (TNF) super family-dependent killing [8].

Although various immune effector cells are recruited to the tumor site, their antitumor functions are largely downregulated in response to several microenvironmental factors. Indeed, hypoxic stress in the tumor microenvironment, which is the result of an inadequate oxygen supply to the cells and tissues, is a characteristic feature of locally advanced solid tumors and is considered as the major mechanism responsible for tumor resistance to therapies [9].

Experimental and clinical evidence indicates that the majority of mechanisms suppressing the antitumor immune functions are directly evolved in the hypoxic tumor microenvironment (reviewed in [10]). Thus, NK cells and natural killer T (NKT) cells infiltrate the tumor microenvironment but are not found in contact with tumor cells [11]. It has been reported that in colorectal, gastric, lung, renal, and liver cancer NK cells appear to predict a good prognosis [12]. However, although they are present in the tumor microenvironment, NK cells may not be able to exert their tumor-killing function. A number of studies reported that NK cells in the tumor stroma have an anergic phenotype that is induced by malignant cell-derived transforming growth factor beta (TGF-β) [13]. Furthermore, immune cells in the tumor microenvironment not only fail to exercise antitumor effector functions but also co-opted to promote tumor growth [14]. In addition, it has become clear that the immune system not only protects the host against tumor development but also sculpts the immunogenic phenotype of a developing tumor and can favor the emergence of resistant tumor cell variants [15]. Thus, it has become obvious that the evasion of immunosurveillance by tumor cells is under the control of the tumor microenvironment complexity and plasticity. Reactivating the immune system for therapeutic benefit in cancer has therefore long been a goal in cancer immunotherapy.

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After decades of disappointment, cancer immunotherapy has recently emerged as a promising treatment of several cancers for which conventional therapies have failed [16]. Notably, the success of the recent proof-of-concept clinical trials of anticytotoxic T-lymphocyte-associated protein 4 (anti-CTLA4) and anti-programmed cell death 1 (anti-PD-1) based on reactivating the adaptive immune response claims that the tide has finally changed. This success is mainly attributed to an increase in our understanding of the mechanisms regulating tumor cell cytotoxicity mediated by immune cells. For several years, our group has been able to participate in this understanding by studying the mechanism responsible for the tumor escape from the immune surveillance [17, 18], which still represents the major obstacle for defining efficient cancer immunotherapeutic approaches. Therefore, it remains important to better understand how tumor cells manage to outmaneuver the immune system and evade effective immunosurveillance.

## 2. Hypoxic stress in the tumor microenvironment

Hypoxia in the tumor microenvironment commonly refers to a condition in tumors where the pressure of oxygen is lower than 5–10 mm Hg. The adaption of tumors to hypoxic stress is regulated by hypoxia inducible factor family of transcription factors (HIFs). It has been demonstrated in a large number of human cancer cases and/or incidents that HIFs were overexpressed and such overexpression is associated with poor response to treatment [19]. Moreover, evidence showed a clear positive correlation between enhanced hypoxic expression of HIFs and mortality [20].

### 2.1. Hypoxia-inducible factors

Three isoforms of HIF have been identified: HIF-1, HIF-2, and HIF-3. HIF-1 and HIF-2 (also known as EPAS1) have the same structure and are well characterized. However, HIF-3 acts as a negative regulator of HIF-1 and HIF-2 [21]. HIF-1 is ubiquitously expressed in all mammalian cells, whereas HIF-2 and HIF-3 are selectively expressed in certain tissues such as vascular endothelial cells, type II pneumocytes, renal interstitial cells, liver parenchymal cells, and cells of the myeloid lineage [22].

HIF-1 is a heterodimer composed of a constitutively expressed subunit and an $O_2$-regulated subunit, HIF-1β, and HIF-1$\alpha$, respectively [23]. In the presence of oxygen, HIF-1$\alpha$ is hydroxylated on a proline residue by prolyl hydroxylase domain protein 2 (PHD2), which leads to an interaction with the von Hippel–Lindau (VHL) protein [24]. This allows the recruitment of an E3 ubiquitin ligase that catalyzes the polyubiquitination of HIF-1$\alpha$ and its subsequent degradation by ubiquitin proteasome system (UPS) [24]. Under hypoxia, the hydroxylation of HIF-1$\alpha$ is inhibited, and HIF-1$\alpha$ is accumulated and translocated to the nucleus where it forms a dimer with HIF-1β and activates the transcription of several genes involved in many biological processes [24]. Similar to HIF-1$\alpha$, HIF-2$\alpha$ is also regulated by oxygen-dependent hydroxylation. While the effect of hypoxia on suppressing the activity of immune cells is relatively well defined, the mechanisms by which hypoxia educates tumor cells to escape an effective immune cell mediated killing are still largely elusive.

## 2.2. Hypoxia-induced autophagy

Although autophagy can be activated in response to different stimuli, including nutrient starvation and/or growth factors withdrawal, hypoxic stress is the major activator of autophagy in the tumor microenvironment [25]. Indeed, emerging recent data have showed that hypoxia-induced autophagy is an important regulator of the innate and adaptive tumor immunity mediated by NK cells and CTL, respectively. In particular, hypoxia has been described to play a central role in activating multiple overlapping adaptive mechanisms involving autophagy and leading to the emergence of resistant tumor cells able to outmaneuver an effective immune response and escape from immune cell killing. In this context, we have recently showed that the activation of autophagy in tumor cells under hypoxia dramatically decreases tumor cell susceptibility to NK- and CTL-mediated lysis [17, 26]. Therefore, autophagy activation is considered to be an important adaptive and resistance mechanism operating in tumor cells to escape the immune system. In accordance with such a role of autophagy, Lotze *et al.* showed that NK cells along with human peripheral blood lymphocytes are primary mediators in inducing autophagy in several human tumors promoting cancer cell survival [27]. Other studies showed that autophagy also plays an important role in regulating CTL-mediated antitumor immune response. While the molecular mechanisms by which autophagy impairs tumor susceptibility to NK and CTL are different, experimental evidence claims that blocking autophagy may improve tumor immunity.

Autophagy is a lysosomal degradation pathway that allows the cell to self-digest its own components, getting rid of excessive or damaged organelles and misfolded proteins in the cell. Such degradation process provides nutrients to maintain crucial cellular functions under nutrient deprivation, thus allowing the survival of cancer cells [28]. It has been reported that the activation of autophagy under hypoxic stress in tumor cells occurs either by HIF-1-dependent or -independent manner. In this section, we will briefly describe the different mechanisms involved in the activation of autophagy.

### *2.2.1. HIF-1-dependent activation of autophagy*

Under hypoxia, HIF-1$\alpha$ is stabilized, and its heterodimerization with HIF-1$\beta$ allows the binding of the transcription factor to hypoxia response elements (HREs) in target genes [9]. HRE is a *cis*-acting hypoxia response element (5′-TACGTGCT-3′), which can be located in either the 5′ or the 3′ regions of the genes [29] to confer oxygen regulation of genes expression [29–32]. The activation of HIF-1$\alpha$-dependent autophagy occurs via the induction of the Bcl-2 (B-cell lymphoma 2)/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), which contains two HRE sites in its promoter region: HRE1 and HRE2. It has been demonstrated that HIF-1 directly binds to HRE2 site in order to induce the expression of BNIP3 [33], thus leading to the disruption of the autophagy inhibitory complex BECN1/Bcl-2 and the subsequent release of Beclin1 (BECN1) to promote the activation of autophagy.

### *2.2.2. HIF-1-independent activation of autophagy*

Despite the role of HIF-1 in the regulation of autophagy under hypoxic conditions, it remains important to note the existence of other pathways that may regulate autophagy under hypoxia independently of HIF-1. Recently, two pathways that influence gene expression and tumor

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cell behavior have been described to be $O_2$ sensitive [34]. One of them occurs through the regulation of the mammalian target of rapamycin (mTOR) and its downstream effectors that orchestrate several biological processes, including autophagy. Indeed, mTOR signaling consists of two major pathways mediated by the specific mTOR complexes mTORC1 and mTORC2. It has been reported that mTORC1 negatively controls autophagy by the inhibition of protein kinase ATG1 involved in the formation of autophagosomes [35]. Hypoxia inhibits mTORC1 through multiple pathways; one of them is mediated through hypoxic activation of the tuberous sclerosis protein (TSC) complex, which is a heterodimeric complex formed by TSC1 and TSC2 [36]. The 5′ AMP-activated protein kinase (AMPK) is a heterotrimeric complex encoded by several genes and is the primary energy sensor in cells. Upon its activation through the increase in the AMP/ATP ratio, AMPK phosphorylates many downstream targets, including TSC2. TSC2 phosphorylation on serine residues 1270 and 1388 enhances the activity of the TSC1/TSC2 complex and thereby blocks the Ras homolog enriched in brain (RHEB)-dependent activation of mTOR [37]. Another pathway that activates autophagy in an HIF-1-independent manner is through the activation of the unfolded protein response (UPR), a program of transcriptional and translational changes that occur as a consequence of endoplasmic reticulum (ER) stress [38]. The UPR is mediated by three ER stress sensors: PKR-like ER kinase (PERK), ER to nucleus signaling 1 (ERN1), and activating transcription factor (ATF) 6 [38]. In some conditions, autophagy appears to be mediated by PERK, whereas in others, it occurs downstream of IRE1. For example, by inducing PERK-dependent phosphorylation of the eukaryotic translation initiation factor 2 alpha (elF2$\alpha$), hypoxia activates autophagy by the transcriptional induction of LC3 through the expression of ATF4 [39].

### *2.2.3. Autophagy activation under nutrient starvation*

Under starvation condition, autophagy is activated by several mechanisms. One of the well described mechanisms is the regulation of autophagy by Reactive Oxygen Species (ROS) which mainly comprise superoxide ($O_2$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical (OH). Several studies have demonstrated that ROS-dependent activation of AMPK leads to the inhibition of mTOR pathway under starvation conditions, thereby activating autophagy [40].

Furthermore, recent studies showed that the upregulation of NS5ATP9 previously identified as p15PAF [proliferating cell nuclear antigen (PCNA)-associated factor] in starved HepG2 cells plays a functional role in starvation-induced autophagy and contributes to tumor cell growth. NS5ATP9 promotes autophagy by Beclin1-dependent manner under starvation condition. Indeed NS5ATP9 upregulates Beclin-1 expression at the transcriptional level, thereby inducing autophagy [41]. Another mechanism of autophagy activation under starvation conditions is MK2/MK3-dependent Beclin1 phosphorylation. MK2/MK3 are two related stress-responsive kinases members of p38 mitogen-activated protein kinase (MAPK) signaling pathway. Yobgjie Wei *et al.* showed that MK2/MK3 phosphorylate Beclin1 at serine 90, and this phosphorylation is essential for autophagy induction in response to nutrient starvation [42].

It has been reported in several studies that some microRNAs (miRNAs) are able to regulate starvation-induced autophagy. Mature miRNAs are a class of noncoding RNAs that play key roles in the regulation of gene expression by acting at the posttranscriptional level. miRNAs are short, single-stranded RNA molecules ~22 nucleotides in length. They are partially complementary to one or more messenger RNA (mRNA) molecules. By base pairing with

sequences found mainly in the 3 untranslated region (3′ UTR) of specific mRNAs, miRNAs downregulate gene expression by different manners, including translational repression. It has been shown in several studies that some miRNAs are able to regulate starvation-induced autophagy. Thus, miR376A and miR376B have been identified as regulators of autophagy under starvation condition by blocking the expression of the two key autophagy proteins ATG4C and BECN1. The inhibition of ATG4C and BECN1 by miR376A is achieved by directly affecting specific MRE (miRNA response elements) sequences in 3′ UTR region. Another autophagy-related miRNA miR181A was shown to regulate starvation-induced autophagy by regulating ATG5 level containing a MRE on its 3′ UTR region. This regulation was observed in MCF-7 breast cancer cells as well as in Huh-7 liver cancer and K562 chronic myelocytic leukemia cell lines [43].

## 3. Hypoxia-induced autophagy as major regulator of the antitumor immunity

Several lines of evidence highlight that hypoxia modulates both the activity of immune effectors and the response of tumor cells to these effectors. In the following section, we will summarize the effect of hypoxia-induced autophagy on the antitumor immune response mediated by CTL and NK cells. Furthermore, we will discuss how autophagy activation regulates tumor cell plasticity and leads to the emergence of resistant tumor cells able to outmaneuver an effective immune response and escape from immune cell killing.

### 3.1. Hypoxia-induced autophagy impairs CTL-mediated tumor cell killing

Autophagy activation not only enables tumor cells to survive stress conditions during cancer development but also provides them an intrinsic resistance mechanism against antitumor immune response. The first evidence for such a role of autophagy was provided by Noman *et al.* who demonstrated that hypoxic lung carcinoma cells can evade CTL-mediated lysis through autophagy induction [26, 44] (Figure 1). Indeed, the inhibition of autophagy using small interfering RNA (siRNA) directed against ATG5 or BECN1 restored tumor cells sensibility to CTL-mediated lysis. This was correlated with a decrease in the hypoxia-dependent induction of the phosphorylation of signal transducer and activator of transcription (STAT)-3. These results allowed the prediction that blocking autophagy would suppress pSTAT3-dependent survival mechanism making tumor cells more susceptible to CTL attack under hypoxia. Considering the degradation role of autophagy, it is difficult, however, to perceive that autophagy is involved in the stabilization of pSTAT3 under hypoxia. Focusing on the crosstalk between the adaptor protein sequestosome1 (SQSTM1/p62), UPS and autophagy, this study revealed that the induction of HIF-1$\alpha$ has two effects in tumor cells: (i) HIF-1$\alpha$ triggers the phosphorylation of Src, which subsequently phosphorylates the tyrosine residue Y705 of STAT3; (ii) HIF-1$\alpha$ activates autophagy by a mechanism involving the increased expression of BNIP3/BNIP3L and the dissociation of the BECN1/Bcl-2 complex. Autophagy activation results in the degradation of the p62 protein. Knowing that p62 is the receptor/adaptor protein responsible for targeting pSTAT3 to the UPS, the autophagy-dependent degradation of p62 leads to the accumulation of pSTAT3. When autophagy is inhibited in tumor cells, the

degradation of p62 is blocked and therefore p62 accumulates in tumor cells. This accumulation accelerates the UPS-dependent degradation of pSTAT3 [26, 44]. The effect of the autophagy inhibitor hydroxychloroquine (HCQ) was also evaluated *in vivo* in combination with a tyrosinase-related protein-2 (TRP2) peptide-based vaccination strategy. Using a transplantable murine melanoma B16-F10 cell line, evidence has been provided that autophagy is primarily detected in hypoxic areas of the tumor. Inhibition of autophagy in B16-F10 engrafted tumors results in a significant decrease in tumor growth by inducing apoptosis, as revealed by TUNEL staining. These results strongly argue for a role of autophagy in mediating hypoxia tolerance to the immune system. More interestingly, a significant decrease in tumor growth was observed in vaccinated and HCQ-treated group of mice as compared to control and to treatment alone. Although the subcutaneous tumor implantation models have their limits, these results strongly argue that *in vivo* inhibition of autophagy improves the antitumor effect of a TRP2-based vaccine.

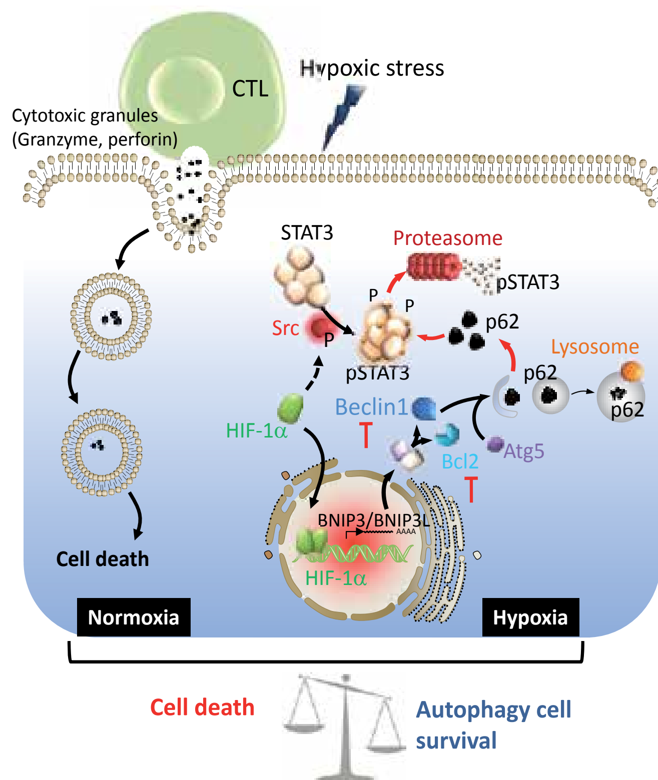


**Figure 1.** Regulation of CTL-mediated tumor cell lysis by hypoxia-induced autophagy. Hypoxic stress leads to the accumulation of HIF-1α. By a yet undefined mechanism, HIF-1α increases the level of phospho-Src, which subsequently phosphorylates STAT3 at the Tyr705 residue. As HIF-target gene products, BNIP3 and BNIP3L are transcriptionally upregulated and compete with the BECN1-BCL2 complex. This competition releases BECN1 from the complex and then activates the autophagic machinery by recruiting several autophagic proteins, including ATG5. As an autophagic substrate, p62/SQSTM1 is degraded in the autophagosomes following their fusion with lysosomes. In view of the fact that p62/SQSTM1 is involved in targeting pSTAT3 to the UPS, its degradation leads to the accumulation of pSTAT3 in cells. In autophagy-defective cells, p62/SQSTM1 is no longer degraded, and its accumulation accelerates the UPS-dependent degradation of pSTAT3.

### 3.2. Hypoxia-induced autophagy impairs NK-mediated antitumor immune response

Recent evidence described how tumor cells can escape fully functional NK-mediated immune surveillance by activating autophagy under hypoxia [17, 45] (Figure 2). Indeed, NK cells recognize and kill their targets by several mechanisms, including the release of cytotoxic granules containing perforin (PRF1) and serine protease granzyme B (GZMB). It has been recently proposed that PRF1 and GZMB enter target cells by endocytosis and traffic to large endosomes named "gigantosomes" [17, 45]. Subsequently, PRF1 is involved in the formation of pores in the membrane of the "gigantosome," leading to the gradual release of GZMB and the initiation of apoptotic cell death. The formation of amphisomes following the fusion between autophagic vacuoles and early endosomes appears to be necessary in some cases for the generation of autolysosomes. In this report [17], the authors described that the proapoptotic protein GZMB is selectively degraded upon autophagy activation in hypoxic cells thereby inhibiting NK-mediated target cell apoptosis.

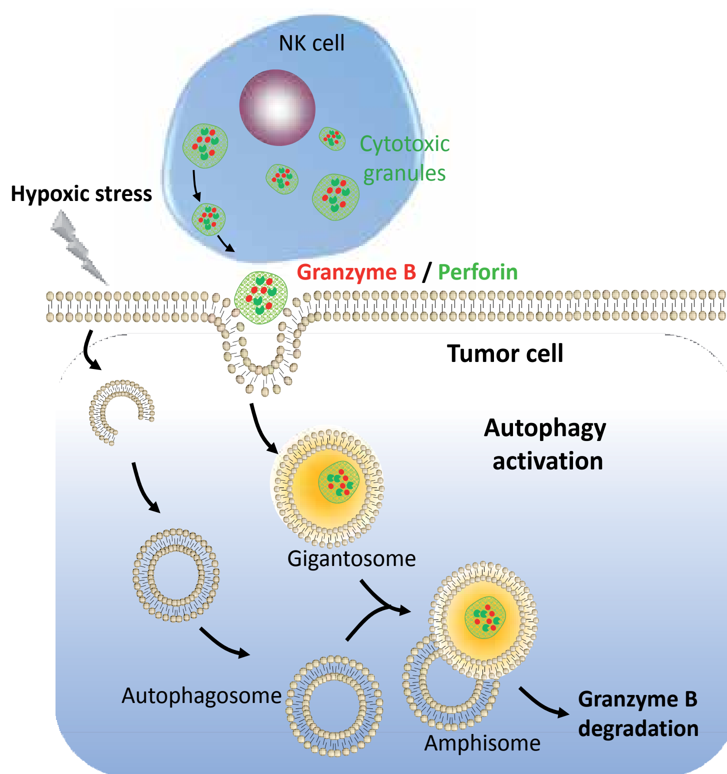


**Figure 2.** Regulation of NK-mediated tumor cell lysis through selective degradation of NK-derived GZMB by autophagy in hypoxic tumor cells. Following the recognition of their targets NK cells secrete cytotoxic granules containing perforin, granzyme B, and other hydrolytic enzymes to the target cells. These granules enter target cells and traffic to enlarged endosomes called "gigantosomes." Under hypoxia, excessive autophagy in target cells leads to the fusion of autophagosomes with gigantosomes containing perforin and granzyme B and the formation of amphisomes. The selective degradation of granzyme B by autophagy leads to hypoxic tumor cell escape from NK-mediated killing.

In light of the *in vitro* observations, they investigated whether targeting autophagy enhances *in vivo* NK-mediated antitumor immune response. BALB/c and C57BL/6 mice were transplanted with syngeneic murine 4T1 breast adenocarcinoma and B16-F10 melanoma tumor cells, respectively. They first demonstrated that NK cells control *in vivo* B16-F10 and 4T1 tumor development as the depletion of host NK cells significantly increases tumor growth. There is a significant decrease of autophagy-defective B16-F10 and 4T1 tumors volumes presumably as a consequence of potentiation of tumor cell killing by NK cells. Overall, this study underlines the inhibition of autophagy as a cutting-edge approach to overcome the suppressive effect of the hypoxic tumor microenvironment on the antitumor immune response.

More recently, the role of autophagy in regulating the NK-mediated immune response was extended to other tumor models. The clear cell renal cell carcinoma (ccRCC) is frequently associated with tumor suppressor VHL gene mutations. Such mutations lead to the stabilization and accumulation of HIF-1$\alpha$ and HIF-2$\alpha$ and their target genes (Figure 3) Using VHL-mutated-786-O renal carcinoma cells, it has been reported that the subsequent stabilization of HIF-2$\alpha$ was strikingly associated with the resistance of 786-O cells to NK-mediated lysis. Targeting HIF-2$\alpha$ or reconstitution of wild-type VHL in 786-O cells (hereafter referred to as WT-7 cells) significantly decreased the level of HIF-2$\alpha$ and restored the resistance of 786-O cells to NK-mediated lysis. These results highlight the critical role of HIF-2$\alpha$ in activating an intrinsic mechanism that makes renal cell carcinoma (RCC) less sensitive to NK cell attack. To gain further insight into the mechanism by which HIF-2$\alpha$ regulates RCC susceptibility to NK-mediated lysis, global gene expression profiling was performed on control and siRNA-HIF-2$\alpha$-transfected 786-O cells. The result showed that the gene inositol 1,4,5-triphosphate receptor, type I (ITPR1) was overexpressed in 786-O as compared to HIF-2$\alpha$-defective cells. Interestingly, targeting ITPR1 in 786-O was sufficient to dramatically restore NK-mediated lysis of these cells. These findings predict that the accumulation of HIF-2$\alpha$ in VHL-mutated 786-O cells leads to the overexpression of ITPR1 which subsequently alters the susceptibility to NK cell attack. Chromatin immunoprecipitation experiment further showed an HIF-2$\alpha$ enrichment of the ITPR1 promoter fragment containing HRE-7 in 786-O compared to WT-7 cells indicating that ITPR1 is a direct target of HIF-2$\alpha$. Interestingly, immunochemistry analysis showed a positive correlation between ITPR1 and HIF-2$\alpha$ expression in RCC patients. They next analyzed whether the accumulation of ITPR1 in 786-O cells was associated with the induction of autophagy. The authors were not able to detect any difference in the activation of autophagy in VHL-mutated 786-O and VHL-corrected WT-7 cells cultured without NK effectors. However, when co-cultured with NK cells, only VHL-mutated 786-O cells were able to activate autophagy. These data strongly argue that the expression of ITPR1 is prerequisite for the induction of autophagy in RCC by a signal derived from NK cells. This was further supported by our data showing that targeting ITPR1 in 786-O cells abrogates the ability of NK cells to activate autophagy [46, 47].

As discussed above, the activation of autophagy in target tumor cells impairs NK-mediated tumor cell killing by degrading NK-derived GZMB. In accordance with this, higher level and activity of NK-derived GZMB were detected in WT-7 as compared to 786-O cells exhibiting increased level of autophagy. Targeting BECN1 in 786-O cells significantly restored GZMB

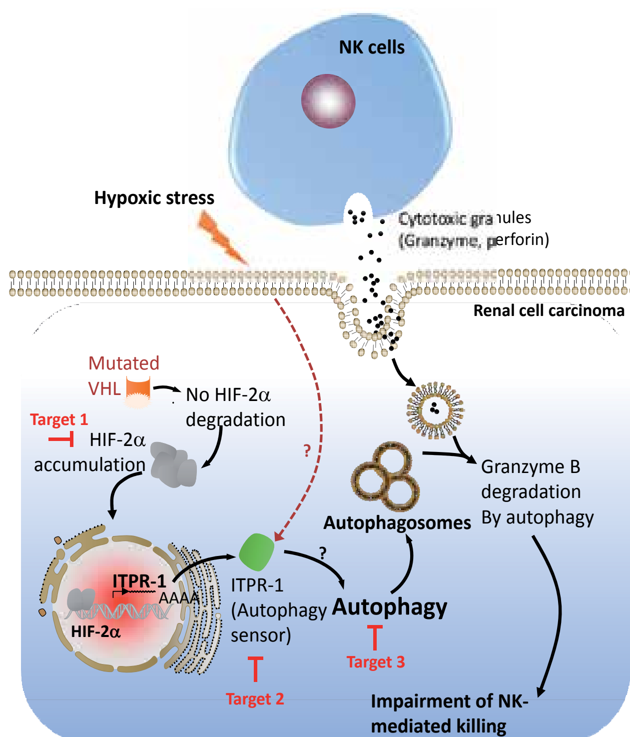


**Figure 3.** Targeting the autophagy sensor ITPR1 in renal cell carcinoma improves NK-mediated tumor cell killing. The expression of mutated VHL in renal cell carcinoma leads to the accumulation of HIF-2*α*. Accumulated HIF-2*α* translocates to the nucleus and induces the transcription of its target gene ITPR1. ITPR1 plays a key role in sensing a yet undefined signal derived from NK cells to activate autophagy by a mechanism that is not fully understood. The activation of autophagy in renal carcinoma cells leads to the degradation of NK-derived granzyme B and ultimately impairs NK-mediated tumor cell killing. At least 3 targets in this pathway (indicated in the figure as Targets 1, 2, and 3) may improve NK-mediated killing in renal cell carcinoma.

level and activity. In light of our *in vitro* observations, the relevance of HIF-2*α*/ITPR1/autophagy pathway on NK-dependent antitumor immune response using Renca murine RCC was investigated. The authors demonstrated first that NK cells control *in vivo* Renca tumor development, by showing that the depletion of host NK cells significantly increased tumor growth. Furthermore, they observed a significant decrease of tumor volume in mice engrafted with ITPR1-defective Renca cells as compared to control cells. This decrease might be due to the improvement of NK-mediated antitumor immune response. Consistent with this hypothesis, the regression of ITPR1 defective tumors was no longer observed in NK-depleted mice. Taken together, these results suggest that inhibiting ITPR1/autophagy in tumors improves their elimination by NK cells *in vivo*. While several studies claim that autophagy inhibitors could improve anticancer therapies, other reports indicate that the use of autophagy inhibitors may also have negative effect in the context of cancer immunotherapy. This study highlights that targeting the autophagy sensor ITPR1 could be an alternative strategy to improve NK-mediated antitumor immune response in renal carcinoma [46, 47].

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### 3.3. Autophagy activation during epithelial-to-mesenchymal transition and tumor cell plasticity

Epithelial-to-mesenchymal transition (EMT) has become one of the most exciting fields in cancer biology. While its role in cancer cell invasion, metastasis and drug resistance is well established [48, 49], the molecular basis of EMT-induced immune escape remains unknown. EMT is a fundamental process in embryogenesis [50] that allows immobilized epithelial cells to migrate as single cells to localize in different organs. Mechanisms driving EMT in development have also been co-opted by carcinoma cells to promote cell plasticity, invasion, and metastasis [10]. Most carcinoma cells exhibit a spectrum of EMT phenotypes or "epithelial cell plasticity," which is directly linked to histological grading and thus contributes to prognosis, stemness, immune suppression, and development of resistant cell variants [51–53]. Epithelial cells are characterized by a well-defined apico-basal polarity involved in the establishment of junctions between cells [54, 55]. The adhesive receptor E-cadherin is a critical component of adherens junctions, and it is often downregulated during tumor progression. Adherens junctions are thus most likely a major structure implicated in the control of epithelial cell plasticity [56]. Upon exposure to EMT inducers, polarized normal or transformed epithelial cells undergo morphological transition by launching a complex program of transcriptional, translational and posttranslational mechanisms.

So far, the relationship between autophagy and EMT in tumors is not well elucidated and studies addressing this issue in the context of tumor immune response are emerging. Thus, the first evidence showing that the acquisition of an EMT phenotype in breast cancer cells is associated with the induction of autophagy and the escape from T-cell-mediated lysis has been published recently [57] (Figure 4). Indeed, using the breast MCF-7-derived tumor cells that have undergone EMT following overexpression of wild-type SNAI1/SNAIL or the constitutively activated (SNAI1-6SA) protein, or by the acquisition of TNF/TNF-$\alpha$ resistance (2101 cells), the authors showed that EMT transcription factors are not the only way to induce an enhanced phenotypic plasticity resulting in breast cancer cell resistance to CTLs. They also showed that the acquisition of resistance to TNF leads to the induction of EMT and the subsequent resistance to antigen-specific killer cells. It is worth noting that the acquisition of resistance to TNF and the high EMT score of TNF-resistant (2101) cells suggest the existence of a level of complexity in the EMT process in which multiple molecules act together to mediate EMT, rather than the master regulators acting on their own.

Consistent with the role of autophagy as a cell protective mechanism, the authors further investigated whether the activation of the EMT program in tumor cells is associated with the induction of autophagy. The results showed that expression of SNAI1 in breast cancer cells induces an epithelial dedifferentiation program that coincides with a drastic change in cell morphology and the activation of autophagy flux. Interestingly, they found that BECN1 is upregulated in mesenchymal cells compared to epithelial cells.

Although the molecular mechanism by which the EMT program affects the expression of BECN1 remained to be addressed, several lines of evidence indicate that this may be related to SNAI1- or EMT-dependent repression of miRNA(s) involved in modulation of BECN1 expression. Indeed, it has been reported that MIR30A inhibits the expression of BECN1, and

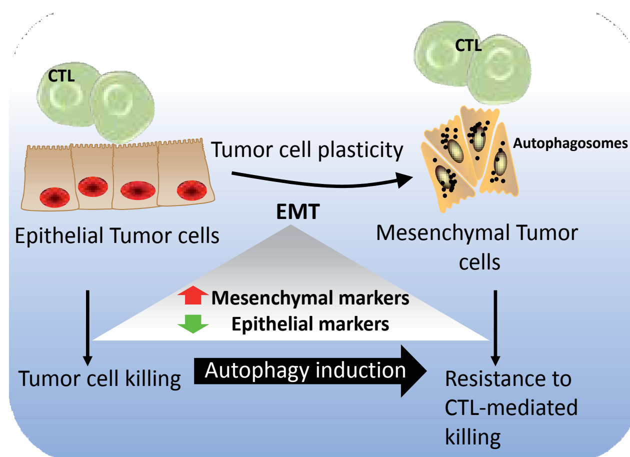


**Figure 4.** The acquisition of an EMT phenotype of tumor cells through loss of epithelial and gain of mesenchymal markers confers resistance to CTL-mediated lysis through autophagy induction.

that the transcription factors SNAI1 and ZEB1 bind to E-boxes in the MIR34A/B/C promoters, thereby repressing MIR34A and MIR34B/C expression. While much remains to be learned mechanistically, this result extended the role of SNAI1 as a regulator of autophagy and paved the way to an interesting topic of research. Although targeting BECN1 in mesenchymal cells is sufficient to restore CTL-mediated tumor cell lysis, it has no effect on cell morphology and the expression of EMT markers. This finding suggests that autophagy is a downstream target of the EMT program in breast cancer cells [57, 58]

## 4. Autophagy as a target for improving anticancer therapies

Intrinsic resistance mechanisms evolved by cancer cells are a key limitation to improve response rates and survival of patients treated with anticancer therapies. It is now clearly established that autophagy activation in cancer cells under stress conditions allows resistance to chemotherapy [59], radiotherapy [60, 61], and immunotherapy [17, 44, 62, 63]. On the basis of these observations, it is not surprising that autophagy has emerged as a potential therapeutic target, and research efforts have intensified to develop autophagy inhibitors that could be used in combination with anticancer therapies.

Pharmacological inhibitors of autophagy identified so far can be classified in two main groups depending on which stage of the autophagy process is targeted. Sequestration inhibitors such as 3-methyladenine (3-MA), LY294002, and wortmannin act at the early stage of the autophagy pathway by inhibiting the class III phosphatidylinositol-3 kinase (PI3K). Recently, a potent small molecule inhibitor of autophagy, called spautin-1, was identified which causes the degradation of the class III PI3K complex by targeting BECN1 [64]. Most of other inhibitory compounds act as later stage. Microtubule poisons such as vinca alkaloids, taxanes, nocoda-

zole, and colchicine cause blockade of autophagosome and lysosome fusion. Inhibitors of lysosomal enzymes (e.g., leupeptin, pepstatin A, and E-64d) or compounds that elevate lysosomal pH (e.g., bafilomycin A1, chloroquine) impair autophagy through the inhibition of cargo degradation by lysosomal hydrolases (reviewed in [28]). Chloroquine (CQ) and its derivative hydroxychloroquine (HCQ) have long been used as antimalarial and antirheumatic drug, and they were the only autophagy inhibitors approved by the US Food and Drug Administration. As single agent, CQ has shown anticancer activity in lymphoma [65], pancreatic [66], and breast cancers [67]. In addition, the use of CQ or HCQ in combination with conventional therapies has provided convincing results in preclinical models [68]. Indeed, autophagy blockade enhances anticancer effects of apoptosis-inducing agents [69] and Src family kinase inhibitors [70]. Moreover, this strategy has shown promising results on patient's survival in the first phase III clinical trial using CQ as adjuvant treatment to conventional anticancer therapy for glioblastoma [71]. Currently, more than 30 clinical trials are registered with the National Cancer Institute to evaluate the effects of autophagy inhibition in a variety of human cancers (http://clinicaltrials.gov). The Table 1 summarize the clinical trials involving CQ or HCQ in combinational treatment of refractory malignancies.

| **Cancer type** | **Drug intervention** | **Phase status** | **Clinical trial ID** | **Title of the clinical trial** |
|---|---|---|---|---|
| Pancreatic cancer | HCQ<br>+Gemcitabine<br>+Abraxane | I/II<br>Active | NCT01506973 | A Phase I/II/Pharmacodynamic Study of Hydroxychloroquine in Combination with Gemcitabine/ Abraxane to Inhibit Autophagy in Pancreatic Cancer |
| Breast cancer | HCQ | II<br>Active | NCT01292408 | Autophagy Inhibition Using Hydrochloroquine in Breast Cancer Patients |
| NSCL cancer | HCQ<br>+Paclitaxel<br>+Carboplatin<br>+Bevacizumab | II<br>Active | NCT01649947 | Modulation of Autophagy in Patients with Advanced/Recurrent Non-small Cell Lung Cancer - Phase II |
| Renal cancer | HCQ+RAD001 | I/II<br>Active | NCT01510119 | Autophagy Inhibition to Augment mTOR Inhibition: A Phase I/II Trial of RAD001 and Hydroxychloroquine in Patients with Previously Treated Renal Cell Carcinoma |
| SCLC | CQ<br>+Chemotherapy<br>+Radiotherapy | I<br>Active | NCT00969306 | Chloroquine as an Anti-Autophagy Drug in Stage IV Small Cell Lung Cancer (SCLC) Patients |
| Colorectal cancer | HCQ+FOLFOX<br>+Bevacizumab | I/II<br>Active | NCT01206530 | FOLFOX/Bevacizumab/ Hydroxychloroquine (HCQ) in Colorectal Cancer |

| Cancer type | Drug intervention | Phase status | Clinical trial ID | Title of the clinical trial |
|---|---|---|---|---|
| Solid tumors | HCQ+Sorafenib | I Active | NCT01634893 | Oral Hydroxychloroquine Plus Oral Sorafenib to Treat Patients with Refractory or Relapsed Solid Tumors |
| Solid tumors | CQ+Carboplatin +Gemcitabine | I Active | NCT02071537 | Chloroquine in Combination with Carboplatin/Gemcitabine in Advanced Solid Tumors |
| Solid tumors | HCQ +Temsirolimus | I Active | NCT00909831 | Hydroxychloroquine and Temsirolimus in Treating Patients with Metastatic Solid Tumors that Have not Responded to Treatment |
| Chronic myeloid leukemia | HCQ +Imatinib mesylate | II Active | NCT01227135 | Imatinib Mesylate with or without Hydroxychloroquine in Treating Patients with Chronic Myeloid Leukemia |
| Pancreatic cancer | HCQ +Gemcitabine +Nab-Paclitaxel | II Active | NCT01978184 | Randomized Phase II Trial of Pre-operative Gemcitabine and Nab Paclitacel with or without Hydroxychloroquine |
| Melanoma, prostate or kidney cancers | HCQ+MK2206 | I Active | NCT01480154 | Akt Inhibitor MK2206 and Hydroxychloroquine in Treating Patients with Advanced Solid Tumors, Melanoma, Prostate or Kidney Cancer |
| Multiple myeloma | HCQ +Cyclophosphamide +Dexamethasone +Sirolimus | I Active | NCT01689987 | Hydroxychloroquine, Cyclophosphamide, Dexamethasone, and Sirolimus in Treating Patients with Relapsed or Refractory Multiple Myeloma |
| Solid tumors | HCQ+Vorinostat | I Active | NCT01023737 | Hydroxychloroquine + Vorinostat in Advanced Solid Tumors |
| Pancreatic cancer | HCQ +Radiotherapy | II Active | NCT01494155 | Short Course Radiation Therapy with Proton or Photon Beam Capecitabine and Hydroxychloroquine for Resectable Pancreatic Cancer |
| Glioma | HCQ +Radiotherapy | II Active | NCT01602588 | A Randomised Trial Investigating the Additional Benefit of Hydroxychloroquine (HCQ) to Short Course Radiotherapy (SCRT) in Patients Aged 70 Years and Older with High Grade Gliomas (HGG) |

| Cancer type | Drug intervention | Phase status | Clinical trial ID | Title of the clinical trial |
|---|---|---|---|---|
| Soft tissue sarcoma | HCQ+Sirolimus | II<br>Active | NCT01842594 | A Phase II Trial of Combined Hydroxychloroquine and Sirolimus in Soft Tissue Sarcoma |
| Melanoma | HCQ<br>+Vemurafenib | I<br>Active | NCT01897116 | A Phase I Trial of Vemurafenib and Hydroxychloroquine in Patients with Advanced BRAF Mutant Melanoma |
| Renal cancer | HCQ+Aldesleukin | I/II<br>Active | NCT01550367 | Study of Hydroxychloroquine and Aldesleukin in Renal Cell Carcinoma Patients (RCC) |
| Colorectal cancer | HCQ+Vorinostat | II Approved | NCT02316340 | Vorinostat Plus Hydroxychloroquine Versus Regorafenib in Colorectal Cancer |
| Advanced cancer | HCQ+Vorinostat or Sirolimus | I<br>Active | NCT01266057 | Sirolimus or Vorinostat and Hydroxychloroquine in Advanced Cancer |
| Prostate cancer | HCQ<br>+Navitoclax<br>+Abiraterone acetate | II<br>Active | NCT01828476 | Navitoclax and Abiraterone Acetate with or without Hydroxychloroquine in Treating Patients with Progressive Metastatic Castrate Refractory Prostate Cancer |
| Melanoma | HCQ<br>+Dabrafenib<br>+Trametinib | I/II<br>Active | NCT02257424 | The BAMM Trial: BRAF, Autophagy and MEK Inhibition in Metastatic Melanoma: A Phase I/2 Trial of Dabrafenib, Trametinib and Hydroxychloroquine in Patients with Advanced BRAF Mutant Melanoma |
| Solid tumors | HCQ<br>+Temozolomide | I<br>Active | NCT00714181 | Hydroxychloroquine and Temozolomide in Treating Patients with Metastatic or Unresectable Solid Tumors |
| Multiple myeloma | HCQ+Bortezomib | I/II<br>Active | NCT00568880 | Hydroxychloroquine and Bortezomib in Treating Patients with Relapsed or Refractory Multiple Myeloma |
| Pancreatic cancer | HCQ<br>+Gemcitabine | I/II<br>Closed | NCT01128296 | Study of Pre-surgery Gemcitabine + Hydroxychloroquine (GcHc) in Stage IIb or III Adenocarcinoma of the Pancreas |
| Colorectal cancer | HCQ<br>+Capecitabine<br>+Oxaliplatin<br>+Bevacizumab | II<br>Closed | | Hydroxychloroquine, Capecitabine, Oxaliplatin, and Bevacizumab in Treating Patients with Metastatic Colorectal Cancer |

| Cancer type | Drug intervention | Phase status | Clinical trial ID | Title of the clinical trial |
|---|---|---|---|---|
| Solid tumors | HCQ +Sunitinib malate | I Closed | NCT00813423 | Sunitinib Malate and Hydroxychloroquine in Treating Patients with Advanced Solid Tumors that Have not Responded to Chemotherapy |
| Multiple myeloma | Rapamycin or HCQ +Cyclophosphamide +Dexamethasone | NS Closed | NCT01396200 | Cyclophosphamide and Pulse Dexamethasone with Rapamycin or Hydroxychloroquine |
| Glioblastoma | HCQ +Radiotherapy +Temozolomide | I/II Closed | NCT00486603 | Hydroxychloroquine, Radiation Therapy, and Temozolomide in Treating Patients with Newly Diagnosed Glioblastoma Multiforme |

**Table 1.** Examples of clinical trials involving autophagy inhibitors in combination with anticancer therapies (http://cancer.gov/clinicaltrials). CQ: chloroquine; HCQ: hydroxychloroquine; NSCL: non-small cell lung; SCLC: small cell lung cancer; NS: not specified.

Despite the encouraging preclinical results supporting the use of autophagy blockers in combination with chemotherapy, more attention should be paid to evaluate the impact of such inhibitors on tumor cell microenvironment. Indeed, recent evidence has emphasized that the cross-talk between cancer cells, and their microenvironment is crucial in determining efficient anticancer immune responses [72, 73]. It is now clear that a potent antitumor immune response is an important prognostic factor for cancer patient overall survival [74], suggesting that simultaneously blocking autophagy in tumor cells and boosting the immune system may be of critical importance to achieve successful anticancer treatment. We recently demonstrated that increased autophagy in tumor cell suppressed the antitumor immune response and that autophagy blockade enhances CTL- and NK-mediated tumor cell killing once they have been activated to lyse tumor cells [17, 63]. Given the limited successes encountered by many immunotherapeutic approaches, these data imply that strategies based on adoptive transfer of T cells, dendritic cell (DC) vaccines, or administration of antibodies or recombinant cytokines such as IL-2, could only be effective if the blockade of autophagy is effective in tumor cells [68]. Indeed, Liang *et al.* showed that the combination of high dose of Interleukin-2 (IL-2) with CQ promotes long-term survival, decreased toxicity, and enhanced immune cell proliferation and infiltration in advanced murine metastatic liver tumor model [75]. This group has now initiated a clinical protocol to evaluate the combinational administration of IL-2 and HCQ in patients with advanced renal cell cancer.

While experimental and preclinical studies were mainly focused on the rational to use autophagy inhibitors in cancer therapy, pharmacological approaches aiming to upregulate autophagy have recently received considerable attention. Accumulating evidence highlights that autophagy plays a crucial role in increasing the immunogenicity of tumor cell and actively participates in tumor-associated antigen processing and presentation [76]. Indeed, cancer cell-associated autophagy contributes to immunogenic cell death (ICD) through the release/

exposure of immunostimulatory danger signals that stimulate the antitumor immune response. Such signaling molecules include secreted ATP, surface-exposed calreticulin, and high mobility group box 1 (HMGB1) release [77, 78]. This important role of autophagy in eliciting ICD was reported in a recent study showing how autophagy-competent cells, but not autophagy-deficient cells, enable to release ATP and recruit dendritic cells and T lymphocytes into the tumor bed in mice [79]. Recently, the same group has confirmed that chemotherapy-induced autophagy in cancer cells determines the outcome of melanoma therapy. Systemic treatment with the anthracycline mitoxantrone reduced the growth of autophagy-competent melanomas but not autophagy-deficient tumors. This growth-inhibitory activity of mitoxantrone observed on autophagy-competent melanomas was shown to be mediated through CD4+ and CD8+ T lymphocytes, suggesting that autophagy is required to trigger a potent anticancer response [80].

Furthermore, it has been described that autophagosomes are essential carriers for cross-presentation of tumor-associated antigens [81]. Li *et al.* have demonstrated that the induction of autophagy in tumor cells, following exposure to alpha-tocopheryloxyacetic acid (alpha-TEA), generates double membrane-bound autophagosomes containing antigens that enhance the cross-priming of CD8+ T lymphocytes. Moreover, the inhibition of autophagy, with 3-MA or by specific silencing of Atg12, partially blocks T-cell activation. The authors showed that vaccination with DC pulsed with autophagosome-enriched fraction, derived from tumor cells treated *in vitro* with alpha-TEA, decreased lung metastasis and increased survival of tumor-bearing mice [82]. Therefore, an autophagy inducer, such alpha-TEA, might be exploited as adjuvant therapy to improve efficacy of immune modulator of T-cell response (anti-CTLA-4 antibody). Moreover, the same group also reported that vaccination with autophagosome-enriched of defective ribosomal products (called DRibbles) or DRibbles loaded onto DC is a potent inducer of the antitumor response in murine cancer models when associated with IFN-$\gamma$ and Toll-like receptor agonist [83]. Based on these findings, a clinical study was initiated to investigate the efficacy of DRibbles vaccine in patients with non-small cell lung cancer. Recently, Amaravadi *et al.* highlighted that cancer patients are suffering from a "systemic autophagic syndrome," meaning that autophagy is activated in tumor cells while suppressed in immune effectors [68]. Taken together, these observations emphasize that future therapeutic approaches may combine *ex vivo* autophagy induction in immune cells and systemic autophagy inhibition to improve efficacy of immunotherapies. As antigen processing and delivery to major histocompatibility complex (MHC) Class I and II molecules into APC is mediated through autophagy cargos, efficient DC vaccines may require their isolation from patients, followed by *ex vivo* activation with tumor-associated antigens, and reintroduction of the matured DC that would facilitate priming of CD8+ T cells.

## 5. Conclusion

The ability of cancer cells to evade immune surveillance and resist immunotherapy raises fundamental questions about how tumor cells survive in the presence of a competent immune system. To address this issue, studies have primarily focused on the mechanisms by which

tumor cells avoid recognition by the immune system without considering the impact of the tumor microenvironment. Thus, despite intense investigation, the relatively modest gains provided by immunotherapy can be in part attributed to the activation of mechanisms suppressing the antitumor immunity. It is now clearly established that the majority of these mechanisms are likely evolved in the local tumor microenvironment. In line with this, it may be more accurate to consider cancer, which was initially thought to be a disease of cells, then of genes and then of genomes, as a disease of the microenvironment. While remarkable and fairly rapid progresses have been made over the past two decades regarding the role of the microenvironment in cancer biology and treatment, our understanding of its actual contribution in tumor resistance to immune cell attack is still fragmented.

Emerging data indicate that, by inducing autophagy, hypoxia in the tumor microenvironment plays key role in mediating tolerance to immune cell attack. Therefore, an understanding of how autophagy plays such a role may allow better understanding of tumor adaptation and evolution, and ultimately lead to improve the efficacy of therapies.

Despite recent advances in our understanding about the role of autophagy in cancer, the emergence of consensual strategy implying autophagy modulators in anticancer therapy is still challenging. Indeed, harnessing autophagy for therapeutic purposes will require careful consideration on whether, when and how autophagy is induced as a prosurvival mechanism, or is recruited to promote cancer cell killing. To date, most of the studies have focused on the impact of autophagy on tumor cells themselves but it should be more accurate to consider autophagy in the context of the tumor microenvironment. It has been increasingly clear that autophagy may influence the cross-talk between cancer and immune cells, leading to either immunoevasion or immunostimulation. Further knowledge on the impact of autophagy in tumor cells as well as in the tumor microenvironment is necessary to tailor therapies that selectively block suppressive mechanisms that impede antitumor response while promoting the antitumor immunity.

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# Targeting the Autophagy Process in Breast Cancer Development and Treatment

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Additional information is available at the end of the chapter

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**Abstract**

Autophagy is a homeostatic process that degrades long-lived or damaged proteins and organelles. By recycling intracellular constituents, it is buffering metabolic stress under starvation conditions. The autophagy role in cancer remains unclear and complicated as it appears to be involved in tumorigenesis, cancer development and treatment outcome in different ways. Autophagy can act as both tumor-promoting and tumor-suppressing agent depending on the stage of cancer progression. During the initiation of cancer, autophagy prevents cells from further DNA damage and genomic instability. It could also be a cell death mechanism in cancer cells with apoptotic defect. Autophagy can also promote tumor growth by facilitating oncogene-induced senescence or protecting tumors against necrosis and inflammation. Once the cancer is formed, autophagy can contribute to tumor progression (by allowing cells to survive in stressful conditions) and metastasis. There is evidence that breast cancer could also be controlled by autophagy. Regulation of this process, correlated proteins and active factors are currently under scientific study in the aspect of breast cancer effective therapeutic strategies.

**Keywords:** Autophagy, breast cancer, tumorigenesis, Beclin-1, miRNA

## 1. Introduction

Breast cancer (BC) is a potentially life-threatening malignant tumor that still causes high mortality among women. One of the mechanisms through which cancer development could

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be controlled is autophagy. This process exerts different effects during the stages of cancer initiation and progression due to the occurring superimposition of signaling pathways of autophagy and carcinogenesis. Chronic inhibition of autophagy or autophagy deficiency promotes cancer due to instability of the genome and defective cell growth as a result of cell stress. However, increased induction of autophagy can become a mechanism which allows tumor cells to survive the conditions of hypoxia, acidosis or chemotherapy. Therefore, in the development of cancer, autophagy is regarded as a double-edged sword. There are different ways of autophagy process control under scientific research for potential therapeutic treatment in anti-cancer strategies.

## 2. The process of autophagy

Autophagy is a process regulated genetically and/or controlled by a group of evolutionarily conserved genes (ATGs; autophagy-related genes). Initially, autophagy was identified as a cell survival mechanism protecting from nutrient deprivation. It ensures homeostasis by maintaining proteins and organelles turnover. Removing excess or damaged intracellular components in response to stress as well as microorganisms allows cells to restrain damage (including genome instability which limits initiation and progression of cancer) and subsequent inflammation. Cellular stress can be caused by a variety of chemical and physical agents like nutrient starvation, pro-inflammatory state, hypoxia, oxidants, infectious agents and xenobiotics [1, 2]. Under the influence of autophagic pathway, biological and morphological changes have been observed [3]. In certain developmental conditions like in cell's response to metabolic stress or under cytotoxic stimuli, autophagy results in a form of cell death described as programmed cell death type II [4].

Currently, over 35 proteins are believed to be essential for autophagy occurrence and progression [5]. The complete macroautophagy (referred to hereafter as autophagy) is generally divided into the following stages: induction, vesicle nucleation, vesicle elongation and completion, docking and fusion, degradation and then recycling [1, 6]. Vesicle nucleation is the initial step in which proteins and lipids are recruited for construction of the autophagosomal membrane. Nucleation consists of the formation of the phagophore or isolation membrane. In mammalian cells, this process is initiated by activation of the class III PI3K/Beclin-1 complex including the core members hVps34/PIK3C3, Beclin-1 (BECN1) and p150. Numerous additional binding partners of this complex function as either positive or negative regulators and include BAX-interacting factor-1 (BIF-1), Atg14L, UVRAG (UV irradiation resistance-associated gene), Ambra1 (activating molecule in Beclin-1-regulated autophagy protein 1) and Rubikon [1, 5, 7-9]. Rubicon (RUN domain Beclin 1-interacting cysteine-rich-containing protein) has also been shown to negatively regulate autophagy. Subsequently, the phagophore is elongated by several ATG proteins. During this elongation step, microtubule-associated protein 1 light chain 3 (LC3)-I is lapidated to LC3-II. Then, the phagophore is maturated primarily upon the action of LC3-II and BECN1 proteins. Maturation leads to the formation of autophagosome (enclosed vesicle). The regulation of the maturation process of the autophagosome is multi-factorial and involves Rab GTPase, SNAREs (soluble N-ethylma-

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leimide-sensitive fusion attachment protein receptors) and ESCRT (endosomal sorting complexes required for transport) proteins, molecules of the acidic lysosomal compartment (e.g. v-ATPase, LAMP proteins- lysosome-associated membrane glycoprteins; lysosomal carriers and hydrolases) and Beclin-1. Finally, the autophagosome fuses with the lysosome to form an autolysosome. The internal material of the autophagic vacuole is degraded by the lysosomal hydrolases.

Basal levels of macroautophagy are kept in check by mTORC1 (mammalian target of rapamycin complex 1) which phosphorylates Atg13 and ULK1 (uncoordinated 51-like kinase 1/ Atg1) or ULK2. This activity in consequence is giving the inhibition of FIP200 (focal adhesion kinase interacting protein of 200 kD/Atg17) phosphorylation by ULK1 [10]. The mTORC1 complex is an important component of a network that accordingly maintains homeostasis by controlling the levels of anabolism and catabolism. For example, high levels of amino acids maintain mTORC1 in an active state by enhancing its binding to regulatory GTPases, Rag (Ras-related GTPase) and Rheb (Ras homolog enriched in brain) [11]. mTORC1 activity could be indirectly induced by insulin and IGF1 (insulin like growth factor 1) [6]. Low glucose levels or high AMP levels (adenosine 5′-monophosphate), indicators of low cellular energy status or stress, could activate AMPK (AMP-activated protein kinase) which in turn inhibits mTORC1 and stimulates autophagy [2,7].

The role of the PI3K/Akt pathway is to suppress autophagy. This pathway activation was shown to decrease autophagy through mTOR activation. It has been considered for cancer treatment. The MAPK pathway also plays a significant role in autophagy. Ras may play a dual role in autophagy [12]. When Ras activates PI3KCA, autophagy is inhibited; however, when it selectively activates the MAPK pathway, autophagy is stimulated.

## 3. Breast cancer

Breast cancer (BC) is the most common and fatal cancer in women worldwide. Decreasing mortality rates can be observed that result mostly from efficient screening strategies [13] but still BC is ranked on the second place in mortality among cancer types [14]. It has been estimated that approximately 1.3 million females develop BC each year with around 465.000 expected to succumb to the disease [15,16]. It is causing death of about 350.000 women in both developed and developing countries every year (with slightly more cases in less developed than in more developed regions) [17]. According to another data presented by DeSantis et al., there are still 500.000 breast cancer deaths per year worldwide [18]. More than 90% of lethality in patients is caused by metastasis and the occurrence of distant metastases (distinct metastatic pattern involving the regional lymph nodes, bone marrow, lung and liver) severely limits the prognosis [19,20]. The 5-year survival rate for patients with BC drops sharply from 98% for individuals with localized disease to 23% for those with metastatic disease (cancer statistics from 2012) [21]. A significant subpopulation of patients with metastasis risk has a median survival time of 18–30 months [22].

In the pathogenesis and progression of BC are involved many factors including genetic, biological and environmental factors as well as a lifestyle [17]. For example, Bcl-2 protooncogene is overexpressed in half of all human malignancies and more than 60% of BC and exerts its oncogenic role by preventing cells from undergoing apoptosis [23]. Still, the disease background is not fully clear because it has been estimated that 75% of women with sporadic invasive BC have no known epidemiological risk factors [24].

## 4. Autophagy regulation in breast tumors development

In tumor genesis and treatment responsiveness, autophagy role is complicated and context-dependent. It presumably differs in different stages of cancer development. At the initial stages, autophagy may represent a protective role thanks to its catabolic functions by degrading and/or recycling cell components (e.g. damaged organelles and misfolded proteins) [25-27]. It also protects against the deleterious effects of ROS (reactive oxygen species) in the cells. Proliferation of cells with cancer-linked mutations may be retarded by autophagy. It can also limit propagation of this type of mutations and consequently suppress tumorigenesis by facilitating the cellular senescence phenomenon (biological aging). However, once a tumor develops, the cancer cells can utilize autophagy for their own cytoprotection and use enhanced autophagy to survive under metabolic and therapeutic stress [27]. Autophagy might increase oxidative stress, hence promoting genome instability and malignant transformation [25-27]. As an example, autophagy has been shown to be required for the transformation of mouse embryonic fibroblasts by the Ras oncogene and this effect is linked to its role in nutrients recycling, such as glucose uptake and increased glycolytic flux [28]. What's more, it has been suggested that metastatic cancer cells can escape from anoikis (process of apoptosis induced by lack of correct cell-ECM attachment) through the autophagy induction [29, 30]. The AMP-activated protein kinase (AMPK) stress response pathway is involved in mediating anoikis resistance by inhibiting mTOR and suppression of protein synthesis. The Ras/MAPK and PI3K/Akt pathways are common mechanisms utilized by cancer cells to evade anoikis. Autophagy is necessary for cancer cells survival in hypoxic conditions during the later stages of *in vivo* tumor formation before the vascularization of tumor takes place [31, 32]. However, the induction of autophagy is associated with cell death in normal cells and in some cancer cells [23]. Autophagic cell death has been described e.g. in anti-estrogen-treated cultured human mammary carcinoma MCF-7 cells [33].Some studies have shown that cancer cells express lower levels of the autophagy-related proteins LC3-II and Beclin-1 than normal epithelial cells. There is evidence that heterozygous disruption of BECN1 promotes tumorigenesis and the overexpression inhibits tumorigenesis, which support the assertion, that defective autophagy or inhibition of autophagy playing a role in malignant transformation [23].

The ability of cancer cells to invade and metastasize is closely correlated with the process of epithelial-mesenchymal transition (EMT). It was recently demonstrated that ectopic expression of the DEDD gene in the MDA-MB-231 metastatic BC cell line led to the degradation of the EMT inducers Snail and Twist through autophagy activation [34]. Reversely, knock-down

of DEDD in the MCF7 non-metastatic BC cell line led to autophagy reduction and EMT promotion [34].

Regulation of autophagy in tumors is governed by principles similar to normal cells only in a much more complicated manner. Abnormal PI3K activation in cancer cells is frequently observed. The multitude of interactions between the PI3K/Akt/mTOR pathway and other cell signaling cascades could often be deregulated [7]. For example, Ras ⁄Raf ⁄ERK pathway, indicated as one of the most commonly deregulated pathways identified in tumors, frequently are observed activating mutations in Ras or B-Raf oncogenes [4]. ERK activity has been associated with autophagy and autophagic cell death in many cellular models in response to different stresses [4]. It also happens in TNFalpha treatment in MCF-7 cells. A deregulated PI3K/Akt/mTOR axis not only suppresses autophagy process but also induces protein translation, cell growth and proliferation thereby can force tumorigenesis. Tumors with constitutively active PI3K mutations, PTEN loss or Akt activation would be expected to be dependent on autophagy for energy homeostasis and survival. Suppression of autophagy by the PI3K cascade is disadvantageous for rapidly proliferating tumor cells and there are theses that compensatory mechanisms (like deregulated apoptosis and/or metabolism) might be concurrently activated to prevent the negative implications of defective autophagy on tumor cell survival.

Many proteins and active factors correlated with autophagy are reported to be associated with human cancers [35]. Various tumor suppressors (e.g. PTEN, TSC1/2, p53, and DAPK) are autophagy inducers whereas some inhibitors of autophagy (e.g. Akt and Ras) possess oncogenic activity [36]. Some studies [37, 38] showed that the more advanced stages of breast cancer over-express several other oncogenic and signaling proteins such as IGF-1R, Cyclin D1, c myc, pERK, Stat3 and Pak4. Some are known activators of Akt/mTOR pathway. Several other autophagy regulators like mitogen-activated kinases (BNIP3) [39] and HSpin 1 (human homologue of the Drosophila spin gene product) [40] play a critical role in cancer cells. Deletion of the essential autophagy gene *FIP200* impairs oncogene-induced tumorigenesis in a mouse model of breast cancer [41].

PTEN, a critical regulator of the PI3K pathway, has a stimulatory effect on autophagy by downregulating PI3K/Akt signaling through inhibition of the Akt/PKB activation. Akt inhibition leads to mTOR signaling suppression and the induction of autophagy [42]. AMPK (AMP-activated protein kinase) pathway has a negative effect on mTOR signaling and promotes autophagy (e.g. upon starvation conditions by activation of Tuberin -TSC2 and/or mTOR signaling inhibitor).

*EI24/PIG8* (etopside induced gene) can be mentioned as another critical factor of autophagic degradation. It remains under control of p53 [43, 44] - well known critical tumor suppressor. p53 effects protectively by cell cycle arrest initiation, removal of cells with incurred DNA damage, senescence and apoptosis. The human EI24 genomic locus is on chromosome 11 in region frequently altered in cancers and was reported to be mutated in aggressive breast cancers. Furthermore, since EI24/ PIG8 (induced by p53) is also known as important apoptotic effector [43, 45], its role may contribute to tumor suppression. EI24/PIG8 loss was associated with tumor invasiveness but not with the development of the primary tumor [45].

mTORC1, class I PI3K, Akt, class III PI3K, Beclin-1 and p53 are critical components of the autophagic pathway that have become major targets of autophagy-related drug design. As an example, rapamycin and its derivatives (e.g. rottlerin, PP242 and AZD8055) target the PI3K/Akt/mTOR signaling pathway to induce autophagy. Spautin-1 and tamoxifen regulate Beclin-1 activity to respectively inhibit and promote autophagy. Oridonin and metformin trigger p53-mediated autophagy and cell death [46].

### 4.1. Beclin-1 role

The most important evidence linking dysfunctional autophagy and cancer come from studies on mice demonstrating that the inhibition of autophagy by disruption of *BECN1* increases cellular proliferation, the frequency of spontaneous malignancies (i.e. lung cancer, liver cancer and lymphomas) as well as mammary hyperplasia. It also accelerates the development of carcinogen-induced premalignant lesions [23]. Additionally, low levels of Beclin1 in human MCF-7 BC cell line can inhibit tumor cell growth [47].

Human breast cancer cell lines FISH analysis with the Beclin-1-containing PAC 452O8 as a probe revealed that 9 out of 22 cell lines had allelic BECN1 deletions [7]. Monoallelic deletion of BECN1 has been also detected in 40-75% cases of human breast, ovarian and prostate tumors [2, 26]. Thereby BECN1 is considered as a tumor suppressor gene [48]. Deletions of Beclin-1 have recently been found mostly associated with BRCA1 in breast and ovarian human tumors (suggesting that BRCA1 loss is the mutation driver and that Beclin-1 is lost because of its proximity to it) [49]. Many breast carcinoma cell lines, although polyploidal for chromosome 17 (*BECN1* gene is placed in 17q21 loci), exhibit deletions of one or more BECN1 alleles. The aberrant expression of Beclin-1 in many kinds of tumors correlates with poor prognosis [26]. Also, heterozygous deletion of BECN1 in mice (BECN1+/−) resulted in increased incidence of spontaneous tumors [48]. Those mice do not have increased incidence of mammary tumors but rather are susceptible to lymphomas and lung carcinomas. BECN1+/− mice tumors express wild-type BECN1 mRNA and protein indicating that Beclin-1 is a haploinsufficient tumor suppressor [7, 8]. The Beclin-1 loss occurring in BC could have important effects independent of autophagy through its interaction with Bcl-2. Bcl-2 is overexpressed in 50%-70% of cancers including BC. An inverse correlation of Beclin-1 and Bcl-2 expression has been described in breast cancer tissue. Bcl-2 expression was correlated with histological grade, tubule formation, nuclear pleomorphism, mitotic count, ER and distant metastasis [49].

Beclin-1 also alters the expression of several autophagy proteins such as Atg5 and UVRAG [26].

### 4.2. Adipokines role

Adipokines, auto-/ endocrine and paracrine-acting bioactive molecules secreted by adipose tissue are one of the recently discovered factors correlating with autophagy and BC [50]. Adiponectin (AdipoQ) is the cytokine secreted in greatest abundance. The prevalence correlate low levels of AdipoQ in the blood circulation with higher BC risk and poorer prognosis. In breast tissue, AdipoQ has a direct anti-carcinogenic effect at the site of tumor growth. This cytokine is potentially capable of regulation of autophagy through AMP kinase (5'AMP-

activated protein kinase) and its activation has been observed in breast cancer cells [50]. Liu and colleagues observed that AdipoQ caused upregulatation of autophagy in MDA-MB-231 cells *in vitro* and *in vivo* in cholesterol induced mammary tumorigenesis [51].

### 4.3. microRNA role

MicroRNAs (miRNAs) are endogenous ~22 nucleotide RNAs that suppress gene expression via messenger RNA (mRNA) cleavage and/or translational repression. Unregulated miRNAs of lymphoma, prostate, lung and breast cancers have been also detected in blood plasma and serum. Circulating miRNAs are currently assessed as proxy biomarkers for BC [52]. There is evidence that miRNAs can influence autophagy process in BC cells at many points. MiR-20a, miR-101, miR-106a/b and miR-885-3p have been reported to have direct possibility of targeting ULK1/2 [53]. Also, miR-155 might target multiple players in mTOR signaling including Rheb, RICTOR (RPTOR independent companion of mTOR) and RPS6KB2 (ribosomal protein S6 kinase). MiR-30a and miR-519a can directly target Beclin-1 causing negative regulation in the autophagic flow thereby resulting in decreased autophagic activity. Action of miR-30a was shown in the *in vitro* study on human BC cell lines MDA-MB-468 and MCF-7 [54] by Zhu *et al*. Tumor cells treatment with the mimic of miR-30a decreased the expression of Beclin-1 mRNA and protein whereas administration of the miR-30a antagomir had opposite effects. Furthermore, high expression of miR-30a blunted the rapamycin-induced autophagy activation [54]. Another miRNA, miR-376b also regulates Beclin-1 and it is also targeting directly Atg4C [9, 55] in MCF-7 cells. The antagomir-mediated inactivation of the endogenous miR-376b results in an increased level of Atg4C and Beclin-1 [56]. MiR-374a and miR-630 can modulate the direct regulation of UVRAG. The tumor suppressive miR-101 could act as a potent inhibitor of basal, etoposide-induced and rapamycin-induced autophagy in MCF-7 cells. Also, the miR-101-mediated inhibition of autophagy sensitized BC cells to 4-hydroxytamoxifen-induced apoptotic cell death and thus miR-101 was suggested to modulate the chemosensitivity of cancer cells [9]. Elevated levels of autophagy due to the progressive loss of miR-101, at least in breast cancer cells, have the potential to trigger cancer cell survival [9, 57]. Three components including STMN1 (stathmin1), RAB5A (ras related protein 5A) and Atg4D have been identified as targets of miR-101 among which the over-expression of STMN1 could partially rescue cells from miR-101-mediated inhibition of autophagy. Previously described RAB5A and STMN1 had uncertain roles in autophagy. RAB5A have been shown to regulate ATG5–ATG12 conjugation in the autophagosome completion while STMN1 plays an important role in cell-cycle regulation [58]. Another miRNA, miR-221/222, might inhibit the cell cycle inhibitor, p27Kip1, a downstream modulator of PI3K/Akt thereby leading to autophagic cell death in HER2/neu-positive primary human breast carcinoma MCF-7 cells. The ectopic expression of miR-221/222 renders the parental MCF-7 cells resistant to tamoxifen [59].

### 4.4. Cancer Stem Cells (CSC) and autophagy

Autophagy is thought to be a critical process for cancer stem cells (CSC) or tumor initiating cell maintenance but the mechanisms through which autophagy supports survival of CSCs

remain poorly understood [60]. The CSC theory proposes that heterogeneity within a tumor is driven by a small population of cells which have ability to differentiate and/or self-renewal, increased membrane transporter activity, anchorage independence and ability to migrate, tumorigenic capacities and pluripotency [61, 62]. Breast cancer follows this model since it has been shown that the CD44+/ CD24 low/-phenotype of cell surface markers (which can be found also in normal stem cells in the breast), have an increased ability to form tumors in immunosuppressed mice than the bulk of the tumor cells. It has been predicted that a quality control mechanism like autophagy is important for maintaining normal and cancer stem cell homeostasis [63]. Maycotte *et al*. have previously reported that a subset of BC cell lines enriched in the triple negative (TN) type is particularly dependent on autophagy for survival even in nutrient rich conditions [49]. This process is regulated by autophagy through modulation of STAT3 activity (often activated in TNBC). STAT3 activity is known to be regulated by IL-6 (interleukin 6) paracrine signaling in breast cancer cell lines [64]. The IL-6/ STAT3 pathway has been also shown to be important for TNBC xenograft growth and breast CSC maintenance [65]. Maycotte *et al*. have found that the pathways most affected by autophagy inhibition were related to stem cells, secretion and epithelial to mesenchymal transition [49]. We also show that autophagy regulates the CD44+/CD24 low/-phenotype and mammosphere formation in both the MCF7 and MDA-MB-468 breast cancer cell lines. Although autophagy regulates IL-6 secretion in both the autophagy dependent (MDA-MB-468) and independent (MCF7) cell lines, autophagy inhibition increased IL6 secretion in MCF7 cells while it decreased it in MDA-MB-468 cells. Decreased mammosphere formation in MDA-MB-468 cells induced by autophagy inhibition was reversed with conditioned media from autophagy proficient MDA-MB-468 cells or with IL-6 treatment. This identifies a mechanism by which autophagy selectively regulates CSC maintenance in autophagy-dependent breast cancer cells. Maycotte *et al*. had used a flow cytometry based assay to analyze CD24 and CD44 staining in cells with different levels of autophagic flux ("autophagic flux" represents the synthesis of autophagosomes, transportation of different substrates and degradation of autophagy inside the lysosome) [49]. In both MCF7 and MDA-MB-468 cell lines, the cells with low autophagic flux had decreased CD24 staining. Cells expressing a shRNA for ATG7 or BECN1 had lower levels of CD24 staining in both cell lines and no changes in CD44 were observed indicating that cells with lower levels of autophagy also have lower levels of CD24 expression.

CSCs are characteristically resistant to conventional anticancer therapy which may contribute to treatment failure and tumor relapse. CSCs exhibit the potential for regeneration which may promote tumor metastasis [66]. Recently, autophagy has been shown to be a critical factor for CSC survival and drug resistance [67].

## 5. Authophagy in anti-breast cancer therapies

There are different ways of autophagy process usage and/or influence recognized according to potential therapeutic treatment in anti-cancer strategies.

Inducing protective autophagy and prosurvival mechanism in human cancer cell lines have been shown in a number of currently used antineoplastic therapies including radiation

therapy, chemotherapy (e.g. doxorubicin, temozolomide and/or etoposide), histone deacelty-lase inhibitors, arsenic trioxide, TNF$\alpha$, IFN$\gamma$, imatinib, rapamycin and anti-estrogen hormonal therapy (e.g. tamoxifen) [12, 23]. In fact, the therapeutic efficacy of these agents can be increased if autophagy is inhibited [23].

The scientific evidence suggests that autophagy leads to cell death in response to several compounds including etoposide, rottlerin, cytosine arabinoside and staurosporine as well as deprivation of growth-factors. A link has been demonstrated between autophagy and related autophagic cell death with usage of pharmacological inhibitors (e.g. 3-MA (3-methyl adenine), CQ (chloroquine), bafilomycin A1 or ammonium chloride) and genetic silencing or knock-down (silencing of *BECN1* and *ATG5, ATG7* and/or *ATG12*) approaches for autophagy suppression. This is connected with **cytoprotective form of autophagy** [68].

Autophagy has also been shown to protect against cellular stress induced by the anti-cancer chemotherapeutic drugs (**nonprotective autophagy**) [68]. The cell is apparently carrying out autophagy-mediated degradative functions but where autophagy inhibition does not lead to perceptible alterations in drug or radiation sensitivity. Furthermore, because autophagy is frequently upregulated in tumors in response to therapy, it may protect the tumors against therapy-induced apoptosis [69]. Gewirtz reported that ionizing radiation could promote autophagy in BC cells in cell culture but autophagy inhibition did not alter sensitivity to radiation [68]. Furthermore, the group showed that chloroquine did not sensitize murine breast tumor cells (4T1) to radiation in an immunocompetent animal model. Based on the results obtained, it was impossible to determine whether radiation had promoted autophagy process or the chloroquine actually effectively inhibited autophagy in the tumor-bearing animals. Supposedly, that the lack of sensitization could be related to findings [5] that autophagy inhibition interferes with the immune system's capability for recognition of the tumor undergoing a stress response.

Such disclosures have led to several clinical trials involving the use of the autophagy flux inhibitors as a combination therapy [70] to radiotherapy efficacy improvement in BC patients. For example, to such inhibitors could be included hydroxychloroquine (HCQ). HCQ is a less toxic version of CQ and the best autophagy inhibitor currently commercially available for clinical trials [71]. Irradiated cancer cells can induce damage in neighboring un-irradiated cells by intracellular gap-junction communication or signals released outside of the cells [72]. Huang *et al*. indicated that radiation-induced senescent MDA-MB- 231-2A cells are secreting multiple cytokines and chemokines including CSF2 (colony stimulating factor; expressed in the highest level), CXCL1(C-X-C motif ligand 1), IL-6 and IL-8 (interleukin 8) [73]. These factors are involved in multiple functions during cancer progression. Autophagy inhibition in MDAMB- 231-2A cells significantly decreased the release of CSF2 suggesting that autophagy plays an important role in promoting the secretion of SASPs (senescence-associated secretory phenotypes). In support of this notion, it has been reported that inhibition of autophagy delays the secretion of several senescence-associated cytokines such as IL-6 and IL-8.

**Cytotoxic autophagy** is the next form of autophagy which should be taken under consideration in the field of cancer treatment. Functionally, this form is associated with a reduction in the number of viable cells and/or reduced clonogenic survival upon treatment [74]. For example,

Bristol *et al*. reported that vitamin D (or the vitamin D analog, EB 1089) can be combined with radiation to promote a cytotoxic form of autophagy in breast tumor cell lines (MCF-7 and ZR-75) [75]. Other research groups also showed that the generation of cytotoxic autophagy may either independently lead to cells death or act as a precursor to apoptosis [76]. Gewirtz identified an additional form of autophagy, termed **cytostatic autophagy**, in non–small cell lung cancer cells (A549 and H460) which was induced in similar conditions to the ones previously described with regards to breast tumor cells [74]. What distinguishes cytostatic autophagy from the cytoprotective form is the failure to detect evidence of cell killing reported in the breast tumor cells [74]. Both Gewirtz and Kroemer's group demonstrated cytoprotective autophagy by radiation alone but the addition of vitamin D or EB 1089 converted cytoprotective autophagy to cytostatic autophagy [74,77]. Kroemer's group observed that the depletion of essential autophagy-relevant gene products such as ATG5 and Beclin-1 increased the sensitivity of human or mouse cancer cell lines (H460, A549 and CT26 cells) to irradiation both *in vitro* (where autophagy inhibition increased radiation-induced cell death and decreased clonogenic survival) *and in vivo* after transplantation of the cell lines into immunodeficient BALB/c nude mice (where autophagy inhibition potentiated the tumor growth-inhibitory effect of radiotherapy) [77].

As Ras⁄Raf⁄ERK pathway belongs to the most commonly deregulated pathways identified in tumors and is currently the target of new antitumor strategies based on the inhibition of upstream ERK regulators. Inhibiting ERK activity in combination therapy with classical antitumor compounds might affect the efficiency of such compounds. For example, in MCF-7 human breast adenocarcinoma cell line such combined therapies with: doxorubicin [78], tamoxifen [79], taxol [80] or Δ Raf1 [81] and, TNF$\alpha$ [82] were used. For example, tamoxifen, the most commonly used antiestrogen, exerts its pharmacological action by binding to estrogen receptor alpha (ER$\alpha$) and blocking the growth promoting action of the estrogen in BC cells. However, the development of antiestrogen resistance has become a major impediment in the treatment of ER-positive BC. It was reported that autophagy plays a critical role in the development of antiestrogen resistance and overexpression of Beclin-1 downregulated estrogenic signaling and growth response [83].

### 5.1. Autophagic genes silencing

Some studies using gene silencing to receive therapeutic effect via cell death induction could represent genetic therapeutic approaches. For example, the Bcl-2 protooncogene (preventing cells from undergoing apoptosis) is overexpressed in half of all human malignancies and more than 60% of BC. Bcl-2 overexpression not only leads to the resistance of cancer cells towards chemotherapy, radiation and hormone therapy but also causes an aggressive tumor phenotype in patients with a variety of cancers [23]. Recent findings suggested that silencing Bcl-2 expression (by siRNA) in MCF-7 cells led to significant autophagic, not apoptotic, cell death [84]. It has been demonstrated that the knockdown of autophagy genes (e.g. *ATG5* and *BECN1*) significantly inhibited both autophagy and cell death induced by Bcl-2 siRNA after a long-term treatment of up to seven days [84]. MCF-7 cells are known to be caspase 3-deficient providing a higher threshold for the induction of apoptosis potentially rendering the auto-

phagic cell death pathway more important. Furthermore, about 45-75% of tumor tissues from BC patients do not have detectable caspase 3 expression [85]. Akar *et al*. reported that doxorubicin predominantly induced autophagy at low doses and apoptosis at high doses [84]. Furthermore, the combination of Bcl-2 siRNA treatment with a doxorubicin low dose enhanced the autophagic response, tumor growth inhibition and cell death. It was the first evidence that targeted silencing of Bcl-2 induction of autophagic cell death in BC cells so a new path for further research on this type of alternative therapeutic strategies.

There are studies connecting autophagy genes profile with BC prognosis. For example, Gu *et al*. separated BC patients into two groups according to the TP53 mutation status in their study [86]. Then, detected the differential gene expression patterns of autophagy-related genes and investigated the association of autophagy with BC prognosis. Using microarray analysis, they identified a set of eight autophagy genes (*BCL2, BIRC5, EIF4EBP1, ERO1L, FOS, GAPDH, ITPR1 and VEGFA*), which were significantly associated with overall survival in breast cancer. This classifier could accurately predict the clinical outcome of BC independently of other classical clinical factors such as age, tumor size, grade, status of lymph nodes, ER status, PR status and ERBB2 status.

### 5.2. Pharmacological approach to the autophagic therapies

In pharmacological approach to the anti-tumor autophagic therapies, the aim is to activate or inhibit autophagy. Many drugs and compounds that modulate autophagy are currently receiving considerable attention [26,35]. For example, autophagy inducers such as rapamycin (mTORC1 inhibitor) [26] and its analogs called rapalogs (such as Everolimus; RAD001) are also often used as tools to study autophagy process [6]. Everolimus was shown to enhance the sensitivity of tumors to radiation by induction of autophagy [6].

Also, natural products are considered as potential anti-cancer candidates being direct or indirect sources of new chemotherapy adjuvants to enhance the efficacy of chemotherapy and/ or to ameliorate its side effects [87, 88].

The more challenging issue is the monitoring of autophagic activity in humans, in tissue and blood samples. It seems to be more important to measure autophagic flux than autophagosome number. However, measurements of autophagic flux in paraffin-embedded tissue samples have been unsuccessful till now and even the detection of endogenous LC3-II (commonly used marker for autophagosomes) is problematic in tissue sections [26]

## 6. Concluding remarks

There has been a tremendous amount of progress in our understanding of the role of autophagy in cancer. But still the molecular mechanisms underlying the regulation of autophagy and the role of autophagy in cancer cells are not fully understood but are progressively revealed. Overall, the data support a dynamic role of autophagy in cancer - both as a tumor suppressor early in progression and later as a pro-tumorigenic process critical for tumor maintenance and

therapeutic resistance. The specification of the autophagic cargo in tumors with increased autophagy is important for understanding the changes in metabolism between normal and malignant cells. Undoubtedly, progress in genomics, proteomics and metabolomics will be helpful in this scope. Induction of autophagic cell death may be an ideal approach in resistant cancers therapies. But most experiments regarding BC are carried out on cell lines *in vitro*. Further, functional investigations of autophagy genes using BC cell lines and animal models will increase our understanding of their roles in determining breast cancer prognosis and could thereby provide clinical strategies for the treatment of breast cancer. The first clinical trials where deliberate autophagy inhibition has been attempted in cancer patients are starting to be reported [89-93]. For instance, autophagy inhibition by HCQ in combination with chemotherapy is currently being evaluated in multiple ongoing clinical trials in patients with solid tumors but we should take into account that autophagic effect is context dependent. While tumor cell susceptibility to autophagy may depend on tumor genotype and the therapeutic agents utilized, data are very limited and it remains unclear whether such new strategies will be clinically beneficial.

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# The Role of Autophagy and Apoptosis During Embryo Development

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Additional information is available at the end of the chapter

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**Abstract**

Programmed cell death (PCD) and cell survival are two sides of the same coin. Autophagy and apoptosis are crucial processes during embryo development of Invertebrates and Vertebrates organisms, as they are necessary for the formation of a new organism, starting from a fertilized egg. Fertilization triggers cell remodeling from each gamete to a totipotent zygote. During embryogenesis, the cells undergo various processes, thus allowing the transformation of the embryo into an adult organism. In particular, cells require the appropriate tools to suddenly modify their morphology and protein content in order to respond to intrinsic and external stimuli. Autophagy and apoptosis are involved in cell proliferation, differentiation and morphogenesis. Programmed cell death is a key physiological mechanism that ensures the correct development and the maintenance of tissues and organs homeostasis in multicellular organisms. PCD has been classified into three types, according to the morphology that the dying cells acquire and the molecular machinery involved: PCD type I or apoptosis; PCD type II or autophagy and PCD type III or necrosis (not involved in physiological development). These different types of cell death have specific features that can be used to be identified and characterized. Apoptosis is a highly conserved, genetically-controlled process through which certain cells destroy themselves. Autophagy is an evolutionarily conserved pathway used by eukaryotes for degrading and recycling various cellular constituents, such as long-lived proteins and entire organelles, that was mainly detected in those tissues where abundant cell death is required. Both autophagy and apoptosis are induced under stress conditions as an adaptive response against stress. Usually, environmental stress cause severe effects on embryonic development. Embryos of different species, exposed to different types of physical or chemical stress, temporarily suspend their development and activate several protective strategies, including PCD II and PCD III. Research has yet to elucidate the interplay between these key processes. Not all types of PCD are always detected in association with a developmental process. Unlike the degeneration of tissues of some invertebrates, the tissues of vertebrates undergo PCD preferentially through an apoptotic mechanisms. In this chapter, we will briefly describe some specific features of apoptotic and autophagic processes. We will focus our attention in some useful model systems of invertebrates and vertebrates organisms, where autophagy and apoptosis occur both in physiological and stress conditions; specifically, we will analyze embryos of: the nematode *Caenorhabditis el-*

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*egans*, the insect *Drosophila melanogaster*, the sea urchin *Paracentrotus lividus*, the fish *Danio Rerio*, the mouse mammalian model, and finally we will consider the differentiation of the male and female embryonic germlines in humans.

**Keywords:** Cell death, apoptosis-autophagy crosstalk, stress, differentiation, embryo model systems

## 1. Introduction

Embryonic development is a dynamic and well-coordinated event that includes cell proliferation, differentiation and death influenced by internal and external signals coming from the microenvironment. Research has yet to elucidate the interplay between autophagy and apoptosis, two processes of programmed cell death, and cell proliferation and morphogenesis in embryos of invertebrates and vertebrates.

Programmed cell death is a key physiological mechanism that ensures the correct development and the maintenance of tissues and organs in multicellular organisms [1]. Similar to apoptosis, autophagy is essential for the development, growth and maintenance of homeostasis. It occurs constitutively at basal levels and appears to be increased as an adaptive response to several intracellular and extracellular stimuli. In both lower and higher eukaryotes, autophagy is a crucial event during embryogenesis. It was proposed that autophagy has a key role in insect metamorphosis, representing a dramatic developmental change associated with widespread cell death and complete disappearance of whole tissues.

In this chapter, we will discuss an emerging research field: programmed cell death and cell survival through apoptosis and/or autophagy under physiological and stressful conditions during the development of invertebrates and vertebrates.

Recently, cell death (CD) has been classified into three types according to the morphology and molecular machinery involved: PCD type I or apoptosis; PCD type II or autophagy; and PCD type III or necrosis, not involved in embryo development [2]. These different types of cell death have specific features that can be used for their identification and characterization. Not all types of PCD are always detected in association with a developmental process. Autophagy is mainly detected in those tissues where abundant cell death is required. Vertebrates' tissues undergo PCD preferentially through apoptotic mechanisms in contrast with the degeneration of tissues in some invertebrates [3].

Although many features are specific among different types of death, some overlapping exists between these different mechanisms. It is noteworthy that this crosstalk often allows the conversion of autophagy into apoptosis or vice versa. Thus, if one pathway is blocked, a cell may still die through a second biological pathway.

### 1.1. Apoptosis

Apoptosis is a cellular phenomenon that orchestrates cell suicide following two main pathways: cytochrome *c* liberation from the mitochondria or activation of death receptors. This

genetically controlled process is highly conserved during the evolution from nematodes to mammals, playing critical roles in both homeostasis and development during the morphogenesis and metamorphosis of invertebrates and vertebrates. Cells undergoing apoptosis show a series of physical and biochemical changes such as plasma membrane blebbing, loss of mitochondrial membrane potential, caspase-activation, DNA fragmentation in distinct ladders and, finally, cell disintegration into apoptotic bodies subsequently engulfed by specialized cells. Phosphatidylserine (PS), a phospholipid normally asymmetrically expressed in the inner leaflet of the plasma membranes in living cells during the final stages of apoptosis, is actively extruded from the internal face of the cell membrane of the dying cell; its exteriorization represents one of the markers that identify the cell as a target for phagocytosis [4] (Figure 1).

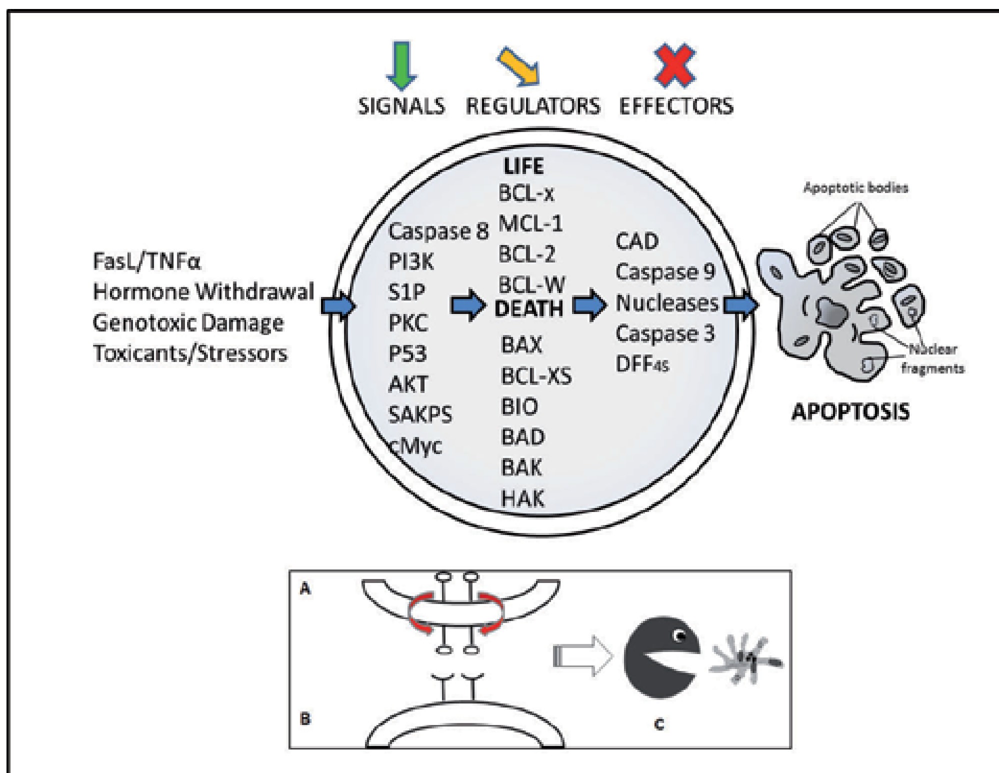


**Figure 1.** *Top*: signals, regulators and effectors involved in the balance between life and death. *Left*, the main inductors of apoptosis. *Bottom*, exemplification of the exposure mechanism of PS from the inner to the outer membrane of an apoptotic cell. (**A**) PS exposure in the apoptotic cell and (**B**) PS receptors of the phagocyte. (**C**) This specific recruitment consents the engulfment of the apoptotic cell or apoptotic bodies and their clearance.

This type of cell death can be greatly affected by ATP levels: if the energy level is not sufficient, cells can undergo partial apoptosis [5]. It is well known that PCD-I is required to remove transitory structures, to sculpt tissues and to eliminate damaged cells that can be harmful to the organism. On the other hand, apoptosis is also employed in response to environmental stimuli to remove cells damaged by chemical, physical and mechanical stress.

### 1.2. Autophagy

Autophagy is an evolutionarily conserved pathway used by eukaryotes for degrading and recycling various cellular constituents such as long-lived proteins and entire organelles [6]. Autophagy, contrary to apoptosis, can induce cell survival or cell death: it is a process of cell survival if the cellular damage is not too extensive; alternatively, it is a process of cell death if the damage/stress is irreversible. In addition, autophagy can act in association with apoptosis or as an independent pathway.

In higher eukaryotes, the lysosome compartmentalizes a range of hydrolytic enzymes and maintains a highly acidic pH in order to decompose into small molecules and then recycle the organelles and the components of cytosol targeted to them [7]. This recycling mechanism allows the cells to conserve their limited resources by minimizing the costs associated with biosynthesis or acquiring resources from the environment.

Depending on how the substrates are delivered to the lysosomal compartment, autophagy is classified into macroautophagy, microautophagy and chaperone-mediated autophagy [8,9]. Macroautophagy involves the formation of autophagosomes that will subsequently fuse with the lysosome. The molecular mechanism governing macroautophagy is highly conserved among eukaryotes. At first, a cup-shaped membranous organelle emerges and encircles a portion of the cytosol which sometimes includes various organelles. This activity results in the formation of spherical bodies, the autophagosomes, in which a double membrane sequesters the compartmentalized material. The outer membrane of autophagosomes fuses with the limiting membrane of lysosomes to release the sequestered materials into the lumen of an autophagolysosome.

In microautophagy, lysosomal compartments engulf a portion of the cytosol along with organelles, forming membrane-bound spherical bodies within lysosomes. In chaperone-mediated autophagy, the substrates, such as proteins, are translocated across the lysosome membrane and delivered directly into the lumen.

In lower eukaryotes, autophagy functions as a cell death mechanism or as a stress response during development. Autophagy's significance and the role (if any) of vertebrate-specific factors in its regulation remain unclear. In particular, in mammals, autophagy may be involved in specific cytosolic rearrangements needed for proliferation and differentiation during embryogenesis and postnatal development. Thus, autophagy is a process of cytosolic "renovation", crucial for cell fate decisions [10]. However, in both invertebrate and vertebrate organisms, it is generally thought that autophagy plays an essential dual role both in the adaptation to stress and in the starvation occurring during morphogenesis, as well as in cell elimination in concert with the apoptotic machinery.

Genetic studies have revealed the importance of autophagy during the early stages of embryogenesis; most of the genes involved, the so-called autophagy-related (ATGs) genes, have been discovered in *Saccharomyces cerevisiae* [11], and their orthologues have been isolated and functionally characterized in higher eukaryotes, indicating that autophagy is an evolutionarily conserved process [12]. Although many apoptosis and autophagy regulatory genes have been

discovered and characterized and some of them are conserved during evolution, the relationship between autophagy and apoptosis still remains rather obscure.

### 1.3. Cell death in stress conditions

Environmental stress can cause severe effects on embryonic development, affecting the phenotype as a result of some emergency responses and adaptive modifications. Embryos of different species, exposed to various toxicants or to physical or chemical stresses, temporarily slow down or suspend their development, eliminating the affected cells throughout apoptosis and thus altering the normal developmental program. In the long run, embryos with several accumulated damages could also die if the stressful conditions persist.

On the other hand, embryos have the ability to activate a general protective strategy against many stress-inducing agents. The accumulation of damaged proteins acts as an inductor signal that activates the stress response and the apoptotic program.

The autophagic process, similar to apoptosis, is triggered as an adaptive response to several intracellular and extracellular stimuli such as toxic stimuli, radiation, nutrient deprivation (starvation), accumulation of misfolded proteins and damaged organelles, hormonal treatments and bacterial/viral infections. Generally, autophagy seems to be crucial for cell survival in stress conditions because it promotes the recycling of damaged proteins and organelles.

A few years ago, we studied both the apoptotic and the autophagic processes in *Paracentrotus lividus* sea urchin embryos, investigating whether these events are activated as a defense strategy after cadmium exposure, a heavy metal recognized as an environmental contaminant [13,14]. Our model suggests that the temporal choice of the apoptotic or autophagic mechanism depends on the persistence of the stressful event: initially, the embryo tries to face the stress conditions using a defense strategy that is less deleterious, namely, autophagy, in an attempt to preserve the developmental program; if this strategy is not sufficient to offset the stress-induced damage, the embryo activates the mechanism of apoptosis. In addition, it can be assumed that autophagy could provide the ATP necessary for apoptosis during development, by recycling damaged cellular components. In light of these and other evidences, it can be hypothesized that there is a close interplay between autophagy and apoptosis [15].

In conclusion, apoptotic and autophagic processes may be used as alternative and/or combined defense strategies by cells exposed to many kinds of stresses. Nowadays, there is a growing interest in cell death via autophagy, which could substitute or act synergistically to the apoptotic pathway.

In this review, we will describe and compare various eukaryotic model systems that use apoptosis and autophagy during development both under physiological and stress conditions. We will also focus on the research methods employed to study the cascade of events involved in these two processes. The purpose of discussing the data in this chapter is not to review all the work in the field but rather to focus on a few arguments with the intent of re-examining some ideas and concepts.

## 2. Apoptosis and autophagy during embryogenesis of eukaryotes

Recent studies have shown that cell death mechanisms are used for specific purposes: morphogenesis during embryogenesis, histogenesis in the progression of metamorphosis and phylogenesis for the elimination of vestigial or larval organs. Like proliferation and differentiation, programmed cell death, PCD-I and PCD-II, play a conspicuous role during normal development as well as during disease conditions. It is essential for the removal of undesirable cells and it is critical both for restricting cell number and for tissue patterning during development.

In both lower and higher eukaryotes, autophagy seems to be crucial during embryo development by acting in tissue remodeling, in parallel with apoptosis. An increase of autophagy is observed in the embryonic stages characterized by massive cell elimination. Moreover, autophagy protects cells during metabolic stress and nutrient paucity occurring during tissue remodeling.

The study of autophagy-defective model systems has highlighted the contribution of PCD-II in the development of invertebrates, for example, during the complex events occurring in the metamorphosis of flies and worms [16]. Furthermore, it has been well documented in the early stages of the development of invertebrates that the activation of apoptotic processes contributes to the formation of different body parts and multiple organs of an organism. Using *Caenorhabditis elegans*, as well as *Drosophila* and mice, it has been demonstrated that developmental cell death is under genetic regulation as shown by mutagenesis experiments.

In vertebrates, on the other hand, there are many examples in which autophagy and apoptosis are involved in embryogenesis. For example, autophagy defects can be lethal for the animal if the mutated gene is involved in the early stages of development or it can lead to severe phenotypes if the mutation affects later stages [17].

Cell death starts at a very early stage in mammalian development. Inhibition of caspase activity leads to the arrest of embryonic development. During gastrulation, apoptosis allows the generation of a pro-amniotic cavity by the removal of the inner ectodermal cells.

Autophagy also has a crucial role during cavitation in the early stages of mammalian development [18]. Furthermore, evidence outlines the importance of autophagy during tissue differentiation in mammals [19].

Both PCD-I and PCD-II are well-controlled biological processes that play fundamental functions during development, differentiation, morphogenesis, tissue homeostasis as well as disease. The different modes of execution of cell death were investigated as separate events from each other. However, in recent times, several findings suggest that these two types of death are often regulated by similar pathways and, depending on the cellular context, can cooperate in a complementary fashion to facilitate cellular destruction. Interactions among components of the two pathways show that there is a complex crosstalk that may be induced by similar stimuli: PCD-I and PCD-II can cooperate, antagonize or assist each other affecting cell fate.

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## 3. Apoptosis and autophagy in the development of the invertebrate model system

### 3.1. Nematodes: *Caenorhabditis elegans*

*C. elegans* has been widely recognized as a suitable model system in developmental research because of some important features: it is a simple and transparent animal with a highly reproducible development and an invariant cell lineage, it is self-fertilizing, it is easy to culture and it has a short reproductive cycle of about 3 days [20]. Recently, it was also recognized as a valuable model organism to study apoptosis and autophagy, two processes redundantly required during its embryogenesis.

Apoptosis can be observed during two stages of *C. elegans* life in two different types of tissues: the "developmental cell death" occurring during embryonic and post-embryonic development of the soma and the "germ cell death" occurring in the gonad of adult hermaphrodites. Developmental cell death can be divided into two moments: between 250 and 450 min after fertilization, the first apoptotic event removes almost a fifth (113/628) of the cells that are generated during embryonic development [21]; the second event occurs during the larval stage L2 and removes some of the newly generated neurons.

During the development of the *C. elegans* hermaphrodite soma, the embryo generates 1090 cells and exactly 131 of these undergo programmed cell death in a highly reproducible manner [22]. It was demonstrated that these dying cells are essentially invariant among individuals since they can be easily identified because of some changes in their morphology and because of their high refractivity under differential interference contrast optics [23]. To unravel the genetic pathway involved in these cell death events, a biochemical characterization of the genes was carried out. The identification of the apoptotic machinery suggested that cell suicide is performed by an evolutionarily conserved molecular program innate in all metazoan cells. Indeed, the key cell death genes of *C. elegans* have one or more mammalian homologs and all the interactions among proteins have also been described for their mammalian counterparts [24].

The first event needed to induce the apoptotic process is the transcription of the egl-1 (egg-laying defective-1) gene coding for a BH3-only protein and directly regulated in a cell-specific manner by transcription factors of the Hox family. This protein will then bind to the Bcl-2 (B cell lymphoma-2)-like anti-apoptotic protein CED-9 (cell death defective-9), which normally protects cells from undergoing apoptosis. This will activate CED-4, the nematode ortholog of the mammalian Apaf-1 [25], which mediates the activation of the caspase CED-3 (CEll Death abnormal) from the inactive zymogen (proCED-3) into the mature protease [26,27]. The activity of CED-4 and CED-3 is essential for the execution of the apoptotic cell process. It is worthy to note that autophagy contributes to the removal of embryonic apoptotic cell corpses by promoting phagosome maturation.

Besides, almost half of the female germ cells undergo apoptosis just before exiting the pachytene stage of meiotic prophase I [28], but egl-1 has no role in this case. During physiological germ cell apoptosis, the nuclei of the apoptotic cells are rapidly cellularized away from

the syncitium, probably to sequester apoptotic factors from the other nuclei. These results indicate that cells in different stages of development within the same organisms are able to trigger different regulatory mechanisms to control programmed cell death [29].

In 2012, *C. elegans* was introduced into the guidelines for the use and interpretation of assays for monitoring autophagy [30]. Numerous studies recognized the advantages of *C. elegans* as a useful multicellular genetic model to study the autophagic pathway. A genetic screen showed the high conservation of the autophagic machinery between yeast and *C. elegans* [31]. It has a single ortholog of most yeast Atg proteins; however, two nematode homologs exist for Atg4 and Atg8.

Autophagy has copious roles during *C. elegans* development. The sperm's entry into the ooplasm triggers the induction of basal levels of autophagy required to remove aggregate-prone proteins and paternally inherited embryonic organelles, i.e. paternal mitochondria and spermatid-specific membranous organelles, by engulfing the autophagosomes and targeting the lysosomes for degradation during early embryogenesis [32]. The localized induction of autophagy around the site of the penetrated sperm indicates that a selective degradation is occurring rather than the bulk degradation of the embryonic cytoplasm [33]. During larval development, autophagy is involved in the specification of multiple cell fates controlled by regulating miRNA-mediated gene silencing through the degradation of one of the components of the miRNA-induced silencing complex, AIN-1 [32].

Autophagy also has an important role during *C. elegans* development under stressful conditions such as starvation, high population density or hyperthermia. *C. elegans* larvae react to these stressful stimuli by arresting development at the third larval stage (L3), an event called the Dauer diapause [34]. Dauer larvae show some metabolic and morphological changes that allow long-term survival, and their cells use autophagy to generate amino acids for the neosynthesis of proteins indispensable for survival during starvation periods [16]. Autophagy was also suggested to be involved in longevity [35,36]. Finally, during the development of the hermaphrodite germline, autophagy acts coordinately with the core apoptotic machinery to execute genotoxic stress [32].

It is worthy to note that, unlike in many other model organisms, programmed cell death is not essential for *C. elegans* viability, at least under laboratory conditions [37]: mutant nematode embryos defective for the core apoptotic cell death pathway are able to develop into adulthood with superficially normal morphology and behavior. Similarly, inactivation or mutations in atg genes do not affect embryonic development and viability. An elegant work [38] showed that, strikingly, double mutant nematode embryos deficient in both autophagy and apoptosis are unable to undergo body elongation or to arrange several tissues correctly. These observations suggested that the apoptotic and autophagic processes are redundantly required and share essential developmental functions for *C. elegans* embryogenesis, revealing why single mutants embryos defective for one of the two processes are able to accomplish the embryonic development [38].

### 3.2. Insects: *Drosophila melanogaster*

*D. melanogaster* provides a powerful model system to investigate both the mechanisms and roles of PCD during animal development with a level of intermediate complexity between nematodes and mammals. The growing appreciation of the conservation of some apoptotic and autophagic responses in insects and mammals is producing an exchange of ideas that is continuing to invigorate this research field.

The development of the *D. melanogaster* embryo into an adult fly is a process that integrates cell proliferation and differentiation with programmed cell death, or apoptosis, according to circumstances. The identification of many central genes required to execute apoptosis provides the tools for the exploration of the phenotypes during development. Genetic manipulation allowed the study of apoptotic regulators and effectors in the context of the whole animal. In *D. melanogaster,* all the embryonic apoptotic processes are initiated by the activity of three genes: reaper, grim and hid. Each of these genes is independently regulated allowing developmental apoptosis to be finely controlled [39]. The role phagocytosis plays in the final stages of apoptosis and the molecular mechanisms guiding the elimination of apoptotic corpses has been outlined [40].

The initiator genes (reaper, grim and hid) in turn activate the core apoptotic machinery, including caspases, a conserved family of cysteine proteases [39]. While caspases have been characterized from many organisms, little is known about insect caspases. In *D. melanogaster*, seven caspases have been characterized, three initiators and four effectors. The availability of new insect genome sequences will provide a unique opportunity to examine the caspase family across an evolutionarily diverse phylum and will provide valuable insights into their function and regulation. This concept is supported by numerous genetic studies in *D. melanogaster,* where a single caspase, Dronc, is essential for most developmental cell death. Importantly, cell death in some cell types occurs in the absence of Dronc and the primary effector caspase, Drice, suggesting that, similar to mammals, redundancies have been built into the cell death system of insects [41].

The methods used to study apoptotic cell death in the *D. melanogaster* embryo and ovary as well as in cultured cell lines have been recently discussed. These techniques, including acridine orange staining, fragmented DNA *in situ* analysis (TUNEL), cleaved caspase staining, caspase activity assays and assays for mitochondrial fission and permeabilization, are suitable for analysis of apoptosis in normal and stress conditions [42].

In *Drosophila*, many developing tissues require programmed cell death (PCD) for proper formation. Two bcl-2 genes are encoded in the *Drosophila* genome and some studies suggested their requirement during embryonic development. However, despite the fact that many tissues in fruit flies are shaped by PCD, deletion of the bcl-2 genes does not perturb normal development. By irradiating fly mutants, it has been demonstrated that developmental PCD regulation does not rely upon the Bcl-2 proteins but that it provides an added layer of protection in the apoptotic response to stress [43].

Cells damaged by environmental insults have to be repaired or eliminated to ensure tissue homeostasis in metazoans. Recent studies on apoptosis induced by stress during embryogen-

esis of *D. melanogaster* demonstrated the implication of Jun N-terminal kinase (JNK) in the apoptotic response to stress. JNK signaling is implicated in many processes of normal development, e.g. the dorsal closure. The repression of Dpp (Decapentaplegic) signalling causes the activation of the proapoptotic role of JNK and the following activation of proapoptotic gene reaper. The protein Schnurri mediates the repression of gene reaper through Dpp. This arrangement allows JNK to control migratory behavior without triggering apoptosis. Dpp plays a dual role during dorsal closure: it cooperates with JNK in stimulating cell migration and also prevents JNK from inducing apoptosis [44].

It has been demonstrated that *Drosophila* lines overexpressing the gene menin or an RNA interference for this gene normally develops but are impaired in their response to several stresses including heat shock, hypoxia, hyperosmolarity and oxidative stress. Menin, the product of the multiple endocrine neoplasia type I gene, is implicated in several biological processes including gene expression control and apoptosis, modulation of mitogen-activated protein kinase pathways and DNA damage sensing or repair. In the *Drosophila* embryo subjected to heat shock, this impairment was characterized by a high degree of developmental arrest and lethality. The gene menin seems to be implicated in the regulation of stress response and in the preservation of protein structure and function, as suggested by the fact that a deletion of the menin gene causes a strong decrease of HSP70 and HSP23 synthesis and an increase of the sensibility to many kinds of stresses [45].

Concerning autophagy, the fruit fly provides an excellent model system for *in vivo* studies in the context of a developing organism. Because of its short life cycle, the well-characterized genetics of the organism and the expression of genes and their regulators, the *D. melanogaster* model system has proven to be very useful in the understanding of the physiological roles of autophagy. Atg genes and their regulators are conserved in *Drosophila* and autophagy can also be induced in response to nutrient starvation and hormones during development. Differences in the role of autophagy in specific contexts and/or cell types suggest that there may be cell context–specific regulators of autophagy and studies in *Drosophila* are well-suited to yield discoveries about this specificity. Autophagy is induced in *Drosophila* in starvation conditions or during metamorphosis in specific tissues. Consequently, the regulation of this process may change in these different contexts and circumstances; thus, this field needs further investigations [46].

During the embryogenesis of *Drosophila*, autophagy and apoptosis seem to occur contemporarily. Autophagy characterizes embryonal districts with massive cell elimination but also exerts a protective role against metabolic stress during tissue remodelling [16].

Analogous to the observations in yeast, worms and mice, Atg inactivation may result in severe phenotypes in *Drosophila*. Mutation of atg18 and atg6 is lethal at the larval stage. Atg1 expression is also crucial for development as atg1 mutants show reduced larval viability and those that survive cannot develop beyond pupa [47]. In Atg7 gene mutant flies, attenuation of autophagy occurs, but there is no impairment of vitality, fertility or morphology. Nevertheless, atg7 mutant adults show higher sensibility to stress and decrease of life span, probably for the neuronal accumulation of ubiquitinated proteins [48].

Induction of autophagy has also been observed during two nutrient status checkpoints of oogenesis in the fruit fly: germarium and mid-oogenesis stages [49]. Mutagenesis experiments in atg7 genes have confirmed these evidences [50]. In *D. melanogaster*, it appears clear that apoptosis and autophagy work in parallel in the disruption of certain tissues during the fly's development. The relationship between autophagic cell death and apoptosis has been examined during *Drosophila* embryogenesis by studying the elimination of an extra-embryonic tissue known as the amnioserosa (AS), demonstrating that both processes are required for the its disruption. Interestingly, autophagy seems to be essential for caspase activation in the AS: a reduction of cell death and permanence of AS are related to the down-regulation of autophagy; a caspase-dependent premature AS dissociation is related to autophagic up-regulation [51].

### 3.3. Echinoderms: *Paracentrotus lividus*

The sea urchin embryo is one of the most important model systems for studies regarding developmental biology, cellular and/or molecular biology and toxicology. In recent years, this organism has become a model for the study of cell death both in physiological and stress conditions. Physiological apoptosis can be considered an important aspect of sea urchin development, which is regulated and controlled by specific genetic programs and is necessary to construct the animal [52].

Studies on the activation of physiological apoptosis in the sea urchin embryo were conducted for the first time in 1997 using different methodological approaches: DNA electrophoresis analysis, morphological observations and TUNEL assay. Physiological apoptosis at pluteus stage (the first larval stage) was shown by those investigations, especially in cells from specific districts: oral and aboral arms and intestine. No apoptotic signals were observed at the blastula or gastrula stage. It has been assumed that some embryonic structures known to at least partially disappear after metamorphosis can be somehow eliminated through this pathway [53].

Further studies showed the occurrence of apoptosis in *P. lividus* larvae with a specific spatial distribution in definite and discrete districts of 8 arms larvae in oral and aboral arms, intestine, ciliary bands, apical and oral ganglia. During the stage of metamorphosis (corresponding to competent and juvenile larvae), only a few cells trigger apoptosis. Therefore, it has been hypothesized that the removal of inadequate cells was the result of a programmed cell death required for the development of the adult and for the elimination of unnecessary structures [54].

Regarding early embryogenesis, the available data suggest that apoptosis is not frequent during developmental cleavage, becoming active from gastrula stages onwards. Only necrotic or pathological cell death has been observed during cleavage stages [55].

The sea urchin embryo, as well as many other embryos of marine organisms, is highly sensitive to several kinds of stressors and is able to activate different defense strategies such as apoptosis. Apoptosis was studied in sea urchin embryos and larvae during stress conditions after exposure to emetine, etoposide [56], ultraviolet radiation [57], staurosporine [58], 12-*O*-

tetradecanoylphorbol-12-acetate (TPA) followed by heat shock at 31°C [53,54], cadmium (Cd) [13, 15, 59, 60], 2-trans-4-trans decadiena [61,62] and chromium (Cr III) [63]. In particular, it was demonstrated that a brief treatment with TPA and heat shock or an exposure to very high Cd concentrations are able to trigger apoptosis in *P. lividus* embryos [53,59]. By TUNEL assays, immunolocalization of cleaved caspase-3, cleaved $\alpha$-fodrin and cleaved lamin-A (two substrates of cleaved caspase-3), an increase of apoptotic events was observed from 15 h to 24 h of development [13]. Other studies showed that long-lasting exposure to lower Cd concentrations, similar to those found in polluted seawaters, caused severe developmental delays and abnormalities in *P. lividus* larvae. The authors reported that small Cd concentrations, if accumulated in the cells, induce several cytotoxic effects and abnormalities depending on cell loss caused by apoptosis. Numerous apoptotic cells could interfere with the developmental program causing a misregulation of cellular remodeling, which normally occurs in premetamorphic stages of development. Probably, the correct larval feeding behavior is perturbed by defects in arms and ciliary bands [60]. In general, apoptosis induced by stress in sea urchin embryos and/or larvae can be considered as part of a defense strategy that, by sacrificing a few cells, can save the whole organism.

Concerning autophagy, the activation of this process in sea urchin embryos was reported for the first time in 2011 [14]. In 2012, the *P. lividus* embryo was included into the "Guidelines for the use and interpretation of assays for monitoring autophagy" as a valuable model system.

Studies on whole embryos make it possible to obtain qualitative and quantitative data for autophagy and also to get information about spatial localization aspects in cells that interact among themselves in their natural environment. In such a system, it is possible to add many autophagic inductors or inhibitors to the media (seawater) that will subsequently be directly absorbed through the membrane of embryo cells [30].

Several experimental approaches have been used to detect physiological autophagy: identification of autophagolysosomes by acidotropic dyes such as neutral red (NR) and acridine orange (AO); immunodetection of LC3-II (an autophagic marker) by Western blotting and immunofluorescence. The results showed that autophagy seems to have a crucial role and it is constantly present, reaching peaks in specific points of embryonic development. This aspect was studied by analyzing the molecular autophagic flux and the dynamics of autophagic organelles (autophagosomes and autophagolysosomes). A major activation of autophagy was detected after 18 h of development, probably because there is a reorganization of the embryo at the gastrula stage as the cells begin to take strategic positions and need to recycle metabolites in order to obtain the energy necessary for the completion of development [14].

Regarding autophagy induced by stress, it has been found that sea urchin embryos are able to modulate this process as a defense strategy against Cd exposure. Analyzing the autophagosomes by LC3, an increase of the levels of this autophagic marker during development was observed in particular at late gastrula stage [14,64]. Specifically, the experiments revealed a higher level of autophagosomes for embryos treated for 18 h with high Cd concentrations, while embryos show lower levels of autophagosomes after 24 h of treatment, probably because the apoptotic process becomes significant [13,14].

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Further studies on the role of autophagy during oogenesis and early stages of development and on the possible interplay between apoptosis and autophagy are in progress in our laboratory.

We proposed three different hypotheses about the homeostatic relationship between survival and death pathways in sea urchin embryos exposed to Cd stress: (a) hierarchical choice of defense mechanisms, (b) energetic hypothesis and (c) clearance of apoptotic bodies. First hypothesis: the embryo tries to face the stress conditions triggering, initially, a less deleterious defense strategy, i.e. autophagy, in order to preserve the developmental program. Second hypothesis: apoptosis is activated since autophagy is unable to offset the damage caused by stress; in this case, autophagy could provide ATP by recycling of damaged cellular components. Third hypothesis: sea urchin embryo begins the clearance of apoptotic bodies through autophagy [14]. To study the functional relationship between autophagy and apoptosis induced by Cd, we blocked autophagy by treatment of the embryos with the inhibitor 3-methyladenine and, subsequently, we analyzed the apoptotic signals. Results demonstrated that the inhibition of autophagy, inevitably, produced a concomitant reduction of apoptosis and the degeneration of the embryos, probably by necrosis.

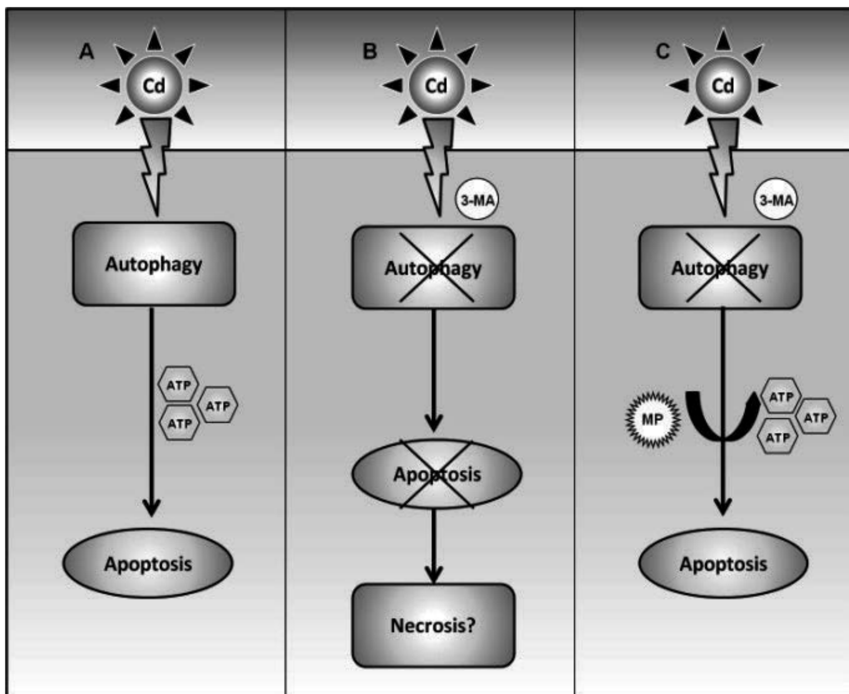


**Figure 2.** Summary diagram on the functional relationship between autophagy, apoptosis and probable necrosis in sea urchin embryos exposed to Cd. (**A**) Cd induces autophagy and then triggers apoptosis. (**B**) Cd-treated embryos, incubated with the autophagic inhibitor 3-MA, do not show autophagy or apoptosis activation and embryos degenerate, probably by necrosis. (**C**) Cd-treated embryos incubated with the autophagic inhibitor 3-MA do not show autophagy but, after incubation with the energetic substrate (MP), there is a re-activation of the apoptotic process.

We hypothesized that, in Cd-exposed embryos, autophagy could operate to supply ATP, recycling damaged cellular components necessary to sustain the apoptotic pathway, which is essential for the clearance of dying cells. This could justify the temporal relationship between autophagy and apoptosis. We showed that by administering an energetic substrate for production of ATP, methyl pyruvate (MP), the apoptotic signals were substantially restored. These data may be explained considering that autophagy could energetically contribute to the apoptotic execution program through its catabolic role [15] (Figure 2).

## 4. Apoptosis and autophagy in the development of the vertebrate model system

### 4.1. Fish: *Danio rerio*

Recently, an increasing number of studies have shown that fish express all the core components equivalent to the mammalian apoptotic machinery, suggesting that at least the central pathways of cell death are highly conserved within vertebrates [65].

Zebrafish (*Danio rerio*) is an exceptional model for developmental research: development is rapid and, thanks to the transparency of the embryo, it is now possible to visualize the apoptotic cells. Thanks to some genetic and bioinformatics screenings, it was demonstrated that zebrafish possesses most homologs of mammalian components of the extrinsic and the intrinsic pathway of apoptosis [66,67]. Since it is supposed that gene and chromosome duplication is a common event in teleosts, several duplicate Bcl-2 family genes were found in zebrafish [68].

In zebrafish embryos, the ability to activate the cell death program is obtained only at gastrulation, simultaneous with the introduction of cell cycle checkpoints, suggesting a close dialog between the cell cycle machinery and the apoptotic machinery [28]. A significant work described the temporal and spatial distribution of apoptotic cells during normal development of the zebrafish embryo from 12 to 96 h after fertilization using a TUNEL assay. The authors found transient high rates of cell death in various structures, focusing on the nervous system and associated sensory organs such as the olfactory organ, retina, lens, cornea, otic vesicle, lateral line organs and Rohon–Beard neurons but also in other non-neural structures such as the notochord, somites, muscle, the vascular and urinary systems, tailbud and fins [69].

Zebrafish also have many features that make it a suitable vertebrate model organism for the analysis of autophagy [70].

To detect autophagic induction, a widely used marker protein is the microtubule-associated protein 1-lightchain 3B protein (MAP1-LC3B) [71]. LC3 is one of the major biochemical markers of autophagy [72]; the MAP (microtubule associated protein) family comprises a group of proteins found in association with microtubules [73] with some possible key roles in interacting with other signaling proteins of the MAP kinase pathway [74]. Both the zebrafish orthologue of mammalian peptide forms, MAP1LC3A (map1lc3a) and MAP1LC3B (map1lc3b), were

identified by phylogenetic and conserved synteny analysis and their expression during zebrafish embryo development was analyzed. Both genes show maternally contributed expression during early embryogenesis; thereafter, levels of map1lc3a transcript steadily increase until at least 120 h post-fertilization. Using the autophagic inhibitor chloroquine, the authors were also able to demonstrate that the LC3II/LC3I ratio increases after the exposure of zebrafish larvae to rapamycin or sodium azide (for mimicry of hypoxia) in their aqueous medium [75].

Because of their aqueous habitat, simple drug administration can be achieved in *in vivo* zebrafish embryo cultures for screening compounds that regulate autophagy and for studying the interplay between genetic and environmental influences on autophagy [76]. The already mentioned transparency of the embryo gives the possibility to observe autophagosome formation by the use of transgenic GFP-LC3. This method was used to study the induction of autophagy in cultured zebrafish embryonic cells under starvation conditions. Analyzing the subcellular localization of endogenous MAP1-LC3B protein during amino acid starvation, the authors found that this protein translocates from the cytosol to the membranes including lysosomes and the endoplasmic reticulum indicating the induction of the autophagy–lysosome pathway [77].

Recent studies indicate that autophagy is one of the major strategies used by marine organisms to face the presence of nanoparticles in the marine environment as the concentration of these emerging contaminants is increasing year by year. Using zebrafish embryos, two papers reported the activation of the autophagy–lysosome pathway after nanoparticle exposure: one of them described the appearance of lysosome-like vesicles after multi-walled carbon nanotubes exposure at the single-cell stage of zebrafish embryos [78]; the simultaneous treatment of embryos with S-doped $TiO_2$ nanoparticles and simulated sunlight irradiation suggested receptor-mediated autophagy and vacuolization indicating the entrance of nanoparticles via endocytosis rather than diffusion [79].

### 4.2. Mammals: Mouse

In mammalian embryos, the apoptotic cell death is mainly implicated in modelling and occurs in processes such as cavitation. In all cases studied, apoptosis is under genetic control, with activation sometimes regulated by local environmental variables [80]. In mammals, a very early activation of zygotic genes and apoptosis occurs at the blastocyst stage, throughout inner cell mass differentiation, differently from most vertebrate in which cell death cannot be seen prior to gastrulation [81,82]. Phosphatidylserine (PS) has been used to identify cell death in the preimplantation of the developing embryo. Annexin V is a very specific apoptotic marker since it preferentially binds to negatively charged phospholipids like PS in the presence of $Ca^{2+}$. Staining is visualized by fluorescence or confocal microscopy or by fluorescence-activated cell sorting. As early as the two-cell stage, cell death was found in the polar bodies. No dying cells in embryos containing 1 to 8 cells were observed, but a few cells died between the 16-cell and blastula stages. Although cell death in the developing preimplantation embryo is not caspase dependent, a generalized inhibition of the caspase activity causes the arrest of

embryonic development even at stages where there is no cell death: the inhibition of caspases causes an ulterior cell death [80,83].

One of the mechanisms responsible for the elevated levels of embryo death during the first week of *in vitro* development, developmental arrest, is often due to ROS. Approximately 10–15% of IVF embryos permanently arrest in mitosis at the 2- to 4-cell cleavage stage, showing no indication of apoptosis. A sensor protein implicated in the apoptotic response to oxidative stress is p66Shc: the deletion of the p66Shc gene in mice leads to a decrease of apoptotic cells and resistance to oxidative stress [84].

Studies on mammalian autophagy suggest the importance of this process in the regulation of cell fate decisions such as differentiation and proliferation. Many autophagy gene knockout mice have embryonic lethality at different stages of development. Furthermore, interactions of autophagy with crucial developmental pathways such as Wnt, Shh (Sonic hedgehog), TGFβ (transforming growth factor β) and FGF (fibroblast growth factor) have been reported. Studying how mammalian autophagy may affect phenotypes associated with development, it was recently shown that knockout of many autophagy-related genes in mice affects early developmental stages, neonatal development and neuronal differentiation [84]. Autophagy is important during critical mammalian developmental stages in which nutrients are restricted, for example, during the preimplantation [85]. Embryogenesis is mainly governed by developmental pathways such as Shh, TGFβ, Wnt and FGF, and there is an intensive crosstalk of autophagic proteins with these pathways. However, it is likely that there are further mechanisms and regulatory loops to be discovered [86].

In mammals, the autophagic process starts just after fertilization. It is known that maternal mRNAs and proteins that accumulate in oocytes during oogenesis are responsible for zygote formation [87]. Following fertilization, maternal proteins and mRNAs are largely degraded, the organelles are remodeled and the translation of zygotic mRNAs starts. Contemporarily, the autophagy seems up-regulated, as demonstrated by a significant increase of autophagosomes. Autophagic activation is probably related to the inactivation of mTOR signalling [86], which occurs after the wave of $Ca^{2+}$ following fertilization. It has been found that the Atg5 protein is essential for the very early developmental stages of mouse. The early induction of autophagy could be necessary for the catabolism of maternal macromolecules and proteins, to obtain a pool of free amino acids to be used for zygotic protein synthesis [88,89]. During the cavitation of mouse embryo, autophagy and apoptosis occur in parallel. The removal of the inner ectodermal cells by apoptosis consents the formation of a proamniotic cavity [18]. Employing embryonic stem cells from embryoid bodies (EB), it has been found that Atg5 and Beclin1 (pro-autophagy genes) are involved in the elimination of cells died in the cavitation mechanism [90]. Recent studies showed the failure of cavitation in EB derived from Atg5- or Beclin-defective cells, probably due to the accumulation of dead cells [91].

In vertebrates, the autophagic process acts as a pro-survival or pro-death mechanism in different physiological and pathological conditions such as neurodegeneration and cancer; however, the roles of autophagy during embryonic development are still largely uncharacterized. Beclin1 is a principal regulator in autophagosome formation and its deficiency results in early embryonic lethality. A functional deficiency of Ambra1, a positive regulator of Beclin1,

is associated with autophagy reduction, increase of ubiquitinated proteins and of apoptotic death and causes serious neural tube defects [92].

A variation of cell proliferation and an impairment of autophagic process always compromises the organ size and often causes a high incidence of tumors [93]. The crosstalk between cell proliferation and differentiation needs further investigations and represents a great objective for the researchers [16].

## 5. Apoptosis and autophagy during differentiation in mammals

There are numerous cases in which the PCD-I and PCD-II are involved during tissue differentiation and cell homeostasis in mammals. For example, correct central nervous system development and neuroretina formation require the activation of the autophagic process [94]. On the other hand, during vertebrate brain development, 20–80% of the originally produced neurons are eliminated by apoptosis [95] and more than 80% of ganglion cells in the cat retina die shortly after birth [96]. It has also been shown that the development of the rat lens vesicle involves the apoptotic elimination of the cells between the ectoderm surface and the optic vesicle helping the invagination and facilitating the separation from the ectoderm [97].

The role of autophagy in cardiogenesis has been carefully investigated demonstrating that constitutive autophagy represents a homeostatic mechanism necessary for cardiomyocyte remodeling, maintaining cardiac size and function. In addition, up-regulation of autophagy plays a protective role for the heart in response to hemodynamic stress increasing protein turnover and preventing the accumulation of abnormal proteins and organelles [98]. Still, in cardiac morphogenesis, apoptosis is essential in generating the overall four-chambered architecture of the heart. The transformation of the endocardial cushion into valves and septa results from a region-specific balance between cellular proliferation, apoptosis and differentiation [99].

A crucial role for apoptosis is seen during morphogenesis and tissue remodeling. The areas of interdigital cell death during limb development provide a paradigmatic model of massive cell death with an evident morphogenetic role in digit morphogenesis. PCD-I sculpts the limbs of all amniotes such as humans, mouse and birds by removal of interdigital webs [100,101]. Cell death can be observed in the anterior and posterior marginal zones of the developing limb bud and in later stages in almost all of the interdigital mesenchymal tissue accompanying the formation of free and independent digits of birds and mammals [102].

### 5.1. From oogenesis to embryogenesis

Autophagy involvement in reproduction has still not been extensively studied, although its activity is fundamental for many processes across the reproduction spectrum from gametogenesis to embryogenesis. Malfunctions in autophagy are associated with deleterious repercussions throughout reproduction.

Death is known to strike the male and female germlines with roughly equal intensity; nevertheless, the innate male germ cells have a major self-renewing ability compared to the female ones. The reproductive life of mammals, including humans, depends on the biological activity of the female gonad that, from birth, has a defined number of oocytes. During a woman's life, most of them are eliminated following a well-defined genetic process [103].

Recent studies on the availability of mice lacking key components of this conserved cell death program were important to confirm the gene expression studies that identified certain molecules as indispensable for female germ cell survival or for death to proceed.

Inadequate nutritional and energy supplies, metabolic stress, hypoxia and growth factor insufficiency are the main inducers of autophagy. The autophagic pathway can also be either promoted or inhibited by cellular components that are involved in the induction of apoptosis. Similarly, autophagy components can also inhibit some players of the apoptotic pathway such as caspase-8 and mitochondria. At both the organism and cell levels, autophagy can (paradoxically) have pro-death or pro-survival functions depending on the context [104].

The activation of the apoptotic pathway in fertilized oocytes is an early event probably responsible for the degradation of superfluous maternal material [105]. This early activation may also be involved in the degradation of organelles derived from spermatozoa involved in fertilization, such as mitochondria, which enter the mammal oocyte after gamete fusion [106]. This autophagic turnover during the early phases of embryo development is known as the oocyte-to-embryo transition, and is a key event for preimplantation embryonic development [87].

Moreover, experimental results showed that autophagy is also activated during folliculogenesis, especially in the granulosa cells [107], as demonstrated by the presence of a strong immunoreactivity for LC3 at all stages of development [108]. By using rat ovaries as a model of follicular development, it has been demonstrated that the induction of autophagy in granulosa cells is closely related to the beginning of apoptosis [108]. In fact, in primordial, primary and pre-antral follicles, caspase 3 is not active. On the contrary, in antral follicles, cleaved caspase 3 signals were detected only in cells that showed a strong immunoreactivity for LC3II, but not in cells expressing an inactive LC3 [109]. Autophagy in granulosa cells seems to be controlled by the levels of FSH, determining the direction of biological processes toward atresia or survival, which is the prerequisite to completing the maturation of the follicle and its ovulation.

Most primordial follicles remain in the quiescent phase during the reproductive life of a woman. Autophagy seems to be regulated by the proto-oncogene KIT through activation of the PI3K-Akt-mTOR pathway. It has been shown in mice that the mutation of the gene results in a significant reduction of the primordial germ cells in the female gonad [110,111]. mTORC1 suppression seems to be the main pathway that is able to support autophagy in the maintenance of primordial follicles in a dormant state throughout a woman's reproductive life [112].

Apoptosis seems to be involved in the gonad's activity. In granulosa cells of secondary and antral follicles, the apoptotic pathway seems to operate in eliminating oocytes with chromatin

defects [113]. Apoptosis during human oogenesis acts on oogonia and oocytes in the preleptotene stage and for the oocytes only in the pachytene stage [114].

Typically, the apoptosis process in mammalian cells is characterized by morphological changes as cytoplasmic or nuclear condensation, apoptotic body formation and chromatin margination along the nuclear membrane. In granulosa cells and oocytes, apoptosis occurs with the segmentation of the oocyte and cytoplasmic vacuolization [115].

Shortly after the fourth week of gestation, primordial germ cells migrate from the yolk sac to the gonadal ridge and proliferate. In the second half of pregnancy, this number declines. Therefore, about $7\times 10^6$ oocytes are formed in the human ovary during early fetal life. This number is sharply reduced before birth through apoptotic cell death of the oocytes. Apoptosis has its highest peak between weeks 14 and 28 and then decreases.

Mitochondria play an important role in the competence of the oocyte to support embryogenesis. It has been demonstrated that to reduce apoptosis, injection into the oocytes of mitochondria derived from other oocytes without an active apoptotic pathway in granulosa cells can be useful [116].

### 5.2. From spermiogenesis to embryogenesis

Autophagy also seems to be represented in the male gametogenesis, and it acts in a way that appears similar to that observed in oocytes [117,118]. An increased focus on autophagy at all stages of gametogenesis appears to be a promising area for future research which has been relatively neglected to date.

The testis produces spermatozoa from spermatogonia in a complex developmental cascade involving proliferation, meiotic maturation and subsequent differentiation of germ cells in the germinal epithelium lining the seminiferous tubules. The duration of this process varies among species.

In the testicle, apoptosis eliminates sperm cells with chromatin or genetic defects, and it regulates the optimal ratio of germ cells to Sertoli cells. It has been speculated that the impairment of apoptosis could be related to the male infertility phenotype [119].

Later in life, apoptosis is involved in the removal of germ cells that are damaged as a result of exposure to environmental toxicants, chemotherapeutic agents or heat [120].

The DNA fragmentation detected in sperm cells can be related to apoptotic events. Normally, "physiological" strand breaks are corrected by a complex process involving H2Ax phosphorylation and the subsequent activation of nuclear poly(ADP-ribose) polymerase and topoisomerase [121]. These strand breaks, achieved by endonuclease-mediated DNA cleavage, could also represent the induction of an incomplete or abortive apoptotic response during spermatogenesis, even if the cell's viability is not compromised.

Malformations and pregnancy loss seems to be influenced by DNA damage in the spermatozoa [122]. In animal models, it has been demonstrated that the genetic integrity of the spermatozoa is closely related to embryonic development and the ability to implant [123].

Apoptosis can be observed in mature spermatozoa as a result of its activation during the spermatogenesis process. In particular, the fragmented apoptotic DNA is easily detected by TUNEL assay or TdT assay (Figure 3).

**Figure 3.** TdT assay: spermatozoa sample. Images captured by fluorescent microscopy in the same field. (**A**) Light, (**B**) fluorescent (green) and (**C**) merged images. Red arrowhead: sperm with flattened head and DNA fragmentation; yellow arrowhead: sperm with angled neck and DNA integrity; blue arrowhead: morphologically normal sperm but with DNA fragmentation; black arrowhead: round cell [124].

## 6. Conclusion

Recent findings have linked the major routes to programmed cell death, apoptosis and autophagy during embryo development, tissue differentiation and homeostatic balance of cells. These distinct types of PCD have been studied separately for a long time; however, recently, accumulated data have suggested that the interaction among components of the two pathways is due to a complex crosstalk. PCD-I and PCD-II are often induced by similar stimuli and even use analogous initiator and effector molecules. Depending on the cellular context, these two main modes of cell death can cooperate in a balanced interplay to facilitate cellular destruction.

In both lower and higher eukaryotes, autophagy seems to be crucial during embryogenesis by acting in tissue remodeling in parallel with apoptosis. An increase of autophagy is observed in those embryonic stages characterized by massive cell elimination.

Moreover, both apoptosis and autophagy have been described as the most important mechanisms that regulating the death of damaged cells in response to stress or in pathologic conditions. Environmental stress can alter embryo development and affect the phenotype as a result of some emergency responses and adaptive modifications which are not the same for all the species analyzed but rather depends on the organism's characteristics. Usually, damage can be restored using different defense strategies but if the cellular insult is beyond the organism's ability to repair, the only alternative can be to destroy the aberrant cell through apoptosis or autophagy.

Here, we have briefly described some specific features of the apoptotic and autophagic processes and their involvement under physiological and stress conditions; we have illustrated

the most remarkable results obtained in some model systems which use apoptosis and/or autophagy for the above-mentioned purposes.

The homeostatic relationship between apoptosis and autophagy during embryo development represents a very interesting chapter, and future studies in this direction are needed to clarify the molecular relevance of the apoptosis–autophagy crosstalk.

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Chapter 6

# Mitophagy Regulated by the PINK1-Parkin Pathway

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Additional information is available at the end of the chapter

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**Abstract**

Mitochondria play key roles in the cellular metabolism of lipids and iron as well as in cell death signaling. Mitochondrial dysregulation produces reactive oxygen species (ROS), which results in oxidative stress. Moreover, the accumulation of damaged mitochondria leads to cell death and tissue dysfunction. Mitochondrial maintenance involves mitophagy, a selective autophagy process that removes abnormal mitochondria. Parkinson's disease (PD) is a movement disorder caused by the specific loss of dopaminergic neurons in the substantia nigra of the midbrain. Two genes implicated in PD, *PINK1* and *Parkin*, regulate mitophagy in cultured cells. Reduction of the $\Delta\Psi m$ leads to activation of PINK1, which stimulates the recruitment of Parkin to the mitochondrial outer membrane of damaged mitochondria and activates Parkin's ubiquitin-ligase activity. Activated mitochondrial Parkin leads to the ubiquitination of mitochondrial proteins and subsequent mitophagy. This elaborate molecular mechanism was recently uncovered and the findings demonstrate the physiological and pathological roles of the PINK1-Parkin pathway. Here, we review these key findings on the molecular mechanism and ideas relevant to neurodegeneration caused by dysregulation of the PINK1-Parkin pathway.

**Keywords:** Dopaminergic neurons, mitochondria, Parkinson's disease, protein kinase, ubiquitin ligase

## 1. Introduction

In eukaryotic cells, mitochondria are highly efficient power-generating systems that perform aerobic respiration. Injured mitochondria leak ROS, resulting in oxidative stress and reduction of energy supply; this dysfunction eventually leads to cell death. Therefore, appropriate regulation of mitochondria is critical for vital activity and anti-aging. Mitochondrial dysregulation has indeed been implicated in various human diseases, including cancer, diabetes, myopathy, and a variety of neurodegenerative disorders such as amyotrophic lateral sclerosis

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(ALS), Huntington's disease, neuropathy, and Parkinson's disease (PD). PD is a progressive neurodegenerative disorder characterized by the degeneration of dopaminergic neurons in the midbrain. The motor symptoms of PD include tremor, rigidity, slowness of movement, and difficulty with ambulation. Although familial forms of PD are relatively rare cases, the identification of genes responsible for PD enables a better understanding of the molecular mechanisms underlying neurodegeneration. *Parkin* and *PINK1* mutations are associated with autosomal recessive forms of early-onset PD [1, 2]. A series of studies on *PINK1* and *Parkin* indicates that these two genes work in a coordinated manner in functions related to mitochondrial maintenance including mitochondria motility, proteasomal degradation of mitochondrial proteins, and selective mitochondrial autophagy (also known as mitophagy). These results strongly imply that dysregulation of mitochondria is one of the major factors in the etiology of PD. In this chapter, we focus on the latest studies that have made significant progress in elucidating the molecular mechanisms of mitochondrial quality control via the PINK1-Parkin pathway.

The selective degradation of mitochondria via an autophagic process was originally reported as mitophagy by J.J. Lamasters et al. [3]. In yeast, loss of the *MDM38* gene product, a component of the mitochondrial protein export machinery, reduces the content of respiratory chain complexes, elicits morphological mitochondrial changes, and disturbs mitochondrial $K^+$ homeostasis, resulting in mitophagy [4]. When mammalian reticulocytes mature into erythrocytes, mitochondria are removed by mitophagy [5]. In fertilized *C. elegans* oocytes, sperm-contributed mitochondria are selectively degraded by mitophagy [6, 7]. These observations indicate that mitophagy plays important roles in mitochondrial maintenance, differentiation, and developmental processes in eukaryotes.

## 2. Mitochondrial segregation and mitophagy

Damaged mitochondria are selectively segregated and degraded by mitophagy [3]. Mitochondrial morphology is maintained by mitochondrial fusion and fission. Mitofusin (Mfn) regulates or mediates mitochondrial fusion whereas Drp1 and Fis1 promote mitochondrial fission in mammals as well as in yeast [8]. Inhibition of Drp1 or Fis1 activity results in the suppression of mitophagy and the accumulation of oxidized mitochondrial proteins, leading to reduced respiration and impaired insulin secretion [9]. Orthologs of *Parkin* and *PINK1* have been identified in *Drosophila* [10–12]. Loss of *Drosophila PINK1* causes mitochondrial degeneration, resulting in male sterility, apoptotic muscle degeneration, and increased sensitivity to multiple stresses, including oxidative stress. Loss of *Drosophila Parkin* produces phenotypes similar to those elicited by the loss of *PINK1*, and Parkin overexpression rescues the mitochondrial defects observed in PINK1 mutant flies [11–13]. The *Drosophila* Parkin and PINK1 phenotypes are suppressed by increased Drp1 activity and are exacerbated by Opa1 or Mfn [14–16]. PINK1 and Parkin collaboratively ubiquitinate Mfn and the steady-state abundance of Mfn is inversely correlated with the activity of PINK1 and Parkin in *Drosophila* [17, 18]. Parkin also ubiquitinates Mfn1 and Mfn2 in mammalian cells, leading to proteasome- and p97/VCP-

dependent degradation [19–21]. These reports indicate that PINK1 and Parkin positively regulate mitochondrial fission, which may facilitate mitochondrial clearance via mitophagy.

## 3. Parkin E3 ligase and ubiquitination

Parkin contains a ubiquitin-like (Ubl) domain at the *N*-terminus, RING-between-RING (RBR) domains at the *C*-terminus, and an atypical RING domain, RING0, in its linker region (Figure 1) [22–24].

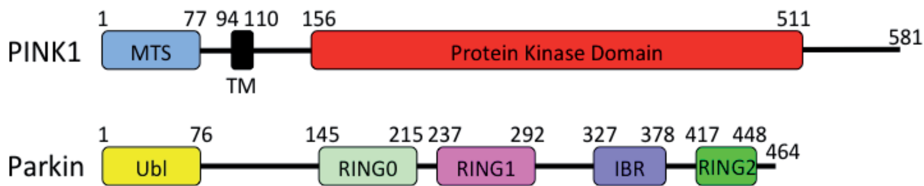


**Figure 1. Schematic of PINK1 and Parkin proteins** MTS: mitochondrial targeting sequence, TM: transmembrane region, Ubl: ubiquitin-like domain, RING: really interesting new gene domain, IBR: in between RING domain. Numbers indicate the positions in the amino acid sequence.

E3 ubiquitin ligases are roughly divided into two groups: RING finger-type E3 ligases and homologous to the E6AP carboxyl terminus (HECT)-type E3 ligases. HECT-type E3 ligases form a thioester intermediate between ubiquitin and a catalytic cysteine residue before transferring ubiquitin from E2 to a substrate. By contrast, RING finger-type E3 ligases mediate the direct transfer of ubiquitin from E2 to the substrate. Parkin, which was formerly classified as a RING finger-type E3 ligase, is now categorized as a HECT-RING hybrid E3-ligase [25, 26]. To activate Parkin, a ubiquitin-charged E2 associates with Parkin RING1 and ubiquitin is transferred from E2 to Cys431 in the RING2 domain of Parkin to form the HECT-like thioester intermediate [25–27]. Similar molecular behaviors were observed in other RBR proteins such as HHARI, and proteins containing a RBR domain are thought to be HECT-RING hybrid E3-ligases [25, 26].

*PINK1* encodes a serine–threonine protein kinase with a mitochondrial targeting signal at the N-terminus (Figure 1) [2]. PINK1 is constitutively processed by mitochondrial proteases at the mitochondrial membrane of healthy mitochondria, resulting in proteasomal degradation [28–30]. The reduction in mitochondrial membrane potential ($\Delta\Psi$m) in damaged mitochondria leads to the accumulation and activation of PINK1 on the outer mitochondrial membrane [29]. Activated PINK1 recruits Parkin from the cytosol to mitochondria in response to decreased $\Delta\Psi$m. This action stimulates Parkin E3 activity, thereby promoting mitochondrial degradation via mitophagy [29, 31–35]. The Ubl domain in the N-terminal region of Parkin inhibits the E3 activity of Parkin by interacting with the RBR region [36]. PINK1 phosphorylates Ser65 in the Ubl domain of Parkin to activate Parkin E3 activity (Figure 2) [37–42]. Activated PINK1 also phosphorylates monomeric ubiquitin at Ser65 in the cytosol. Transient interaction with

phosphorylated ubiquitin leads to a conformational change in Parkin and subsequent activation of Parkin E3 activity [41, 43–45].

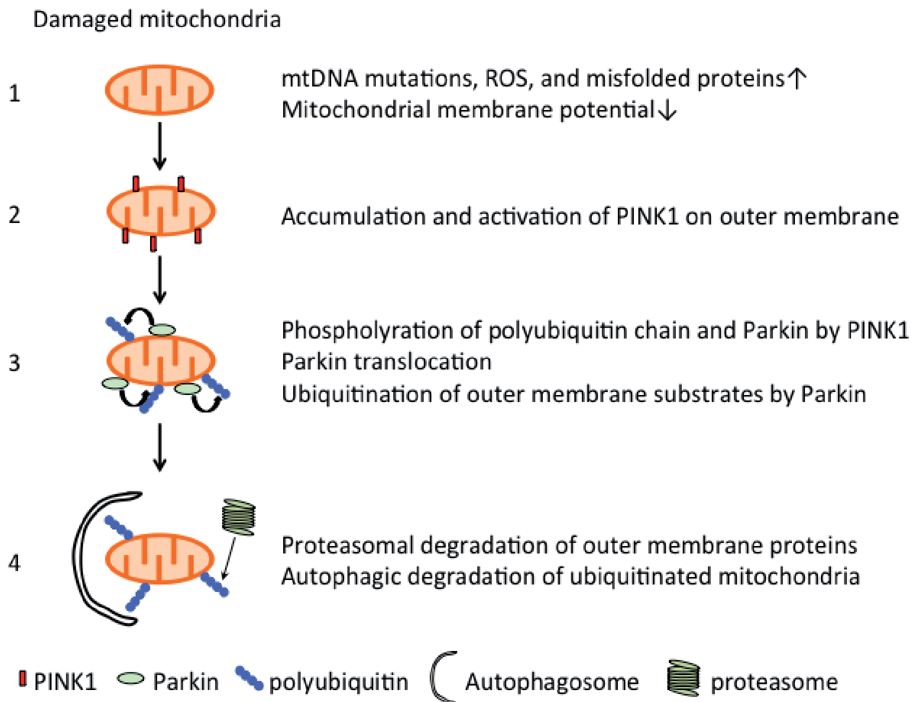


**Figure 2. PINK1-Parkin-mediated mitophagy** (1) Mitochondrial DNA (mtDNA) mutations, ROS overproduction and misfolded protein accumulation cause a reduction in $\Delta\Psi m$. (2) PINK1, which is constitutively degraded under steady-state conditions, accumulates on damaged mitochondria. (3) Accumulated PINK1 activates itself and elicits mitochondrial translocation and Parkin activation through phosphorylation. Activated Parkin ubiquitinates substrates on mitochondria. (4) Polyubiquitinated proteins on the mitochondrial outer membrane are degraded by the proteasome, and damaged mitochondria are eliminated concurrently by mitophagy.

It has been reported that Parkin binds to four tandem-repeated mitochondrial ubiquitin chains, which mimic Lys63-linked polyubiquitin chains only when PINK1 is activated [46]. Subsequent reports have revealed that PINK1 phosphorylates mitochondrial polyubiquitin, resulting in Parkin activation and mitochondrial relocation (Figure 3) [41, 47]. While PINK1 phosphorylates both monoubiquitin and polyubiquitin, including Lys48- and Lys63-linked polyubiquitin chains, activated Parkin preferentially associates with Lys63-linked phosphorylated polyubiquitin chains on mitochondria [41]. Lys48-linked polyubiquitin chains are generally utilized as signals for proteasomal degradation [48], whereas Lys63-linked ubiquitin chains were first identified in yeast as atypical ubiquitin chains that respond to stress [49]. A variety of functions of Lys63-linked polyubiquitin chains were subsequently characterized, including the regulation of kinase activity, DNA damage response, signal transduction scaffolding, vesicular trafficking, and endocytosis [50]. Thus, the formation of Lys63-linked ubiquitin chains during mitophagy might have a critical role beyond Parkin recruitment [51].

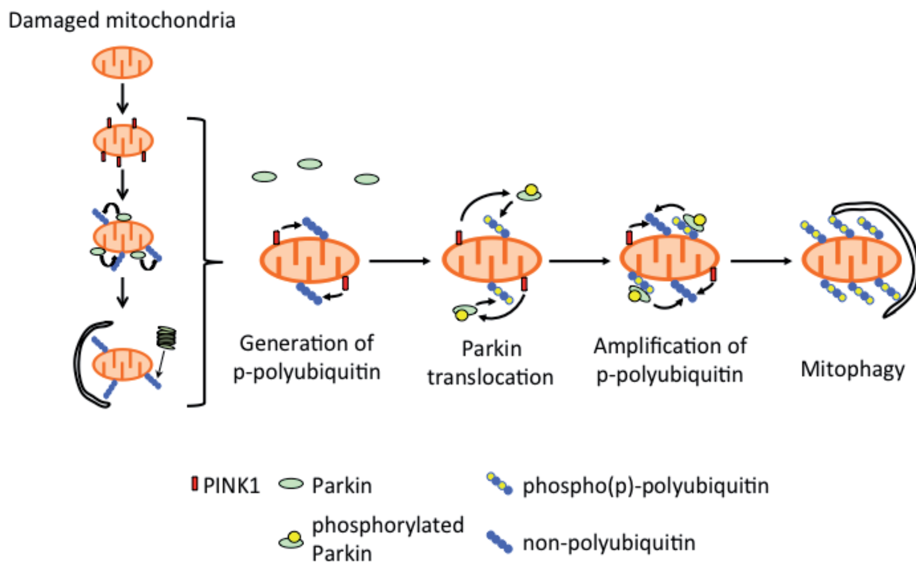


**Figure 3. Amplification of phospho-polyubiquitin chain production achieves rapid Parkin translocation and activation** Phospho-polyubiquitin chains on mitochondria are produced in collaboration with PINK1 and Parkin. The mechanism responsible for the formation of initial ubiquitin chains on mitochondria remains unresolved. The ubiquitin chains might be attached to outer membrane proteins via mitochondrial ubiquitin ligases other than Parkin. Alternatively, Parkin, which is activated by phospho-monoubiquitin in the cytosol, could attach ubiquitin chains to mitochondrial proteins. Phosphorylation of polyubiquitin chains by PINK1 promotes further Parkin activation and relocation to the mitochondrial outer membrane, amplifying the generation of phospho-polyubiquitin chains and subsequently recruiting autophagy machinery to ubiquitinated mitochondria.

Several reports have demonstrated that Parkin-interacting E2 enzymes mediate the ubiquitination reaction of Parkin. UBE2N is related to Parkin-mediated Lys63-linked ubiquitination [51, 52], whereas UBE2N, UBE2L3, and UBE2D2/3 synergistically contribute to Parkin-mediated mitophagy [53]. Knockdown of UBE2N, UBE2L3, or UBE2D2/3 but not UBE2A, UBE2S, or UBE2T significantly reduces autophagic clearance of depolarized mitochondria or Parkin E3 activity [53, 54]. However, recent reports indicate that the linkage property of polyubiquitination depends on Parkin itself rather than involved E2s [47], and that atypical Lys6- and Lys11-mediated polyubiquitination chains are also generated by Parkin and contribute to mitophagy [47, 55].

Deubiquitinating enzymes (DUBs) are also involved in PINK1-Parkin-mediated mitophagy. Because USP30 preferentially removes Lys6- and Lys11-linked ubiquitin chains generated by Parkin on damaged mitochondria, USP30 knockdown rescues the defective mitophagy caused by pathogenic mutations in Parkin [55, 56]. Moreover, knockdown of USP30 improves mitochondrial morphology in *Parkin*- or *PINK1*-deficient flies and protects them from the paraquat-induced reduction in dopamine, motor dysfunction, and shortened lifespan [56]. Conversely, USP8 removes Lys6-linked polyubiquitin on Parkin, which activates Parkin-mediated mitophagy [57]. As USP15 does not affect Parkin autoubiquitination and translocation to mitochondria, knockdown of CG8334, the closest homolog of USP15 in *Drosophila*,

largely rescues the altered mitochondrial morphology and the defective climbing ability in Parkin knockdown flies [58]. These findings could provide new therapeutic strategies for PD via the targeting of DUBs.

PINK1 and Parkin promote selective turnover of the respiratory chain complex in *Drosophila* independently of mitophagy pathway [59]. Quantitative Lys-ϵ-Gly-Gly (diGly) proteomics identified a variety of Parkin-dependent ubiquitinated proteins [47, 60]. Interestingly, the PINK1-Parkin pathway dynamically regulates protein ubiquitination levels in the mitochondrial outer and inner membranes, nucleus, cytoplasm, and cell membrane, suggesting that Parkin affects cellular events other than mitochondrial maintenance. Parkin-mediated xenophagy is one such example although it is unclear whether PINK1 is involved in this mechanism or not [61].

## 4. Regulators of PINK1

PINK1 stability is regulated by mitochondrial outer and inner membrane proteins, and upregulation of these proteins triggers PINK1 activation through dimerization and autophosphorylation (Figure 4) [62, 63]. PINK1 interacts with the translocase of the outer membrane (TOM) complex and is imported to the mitochondrial inner membrane [64, 65]. Under steady-state conditions, endogenous PINK1 is constitutively and rapidly degraded by the E3 ubiquitin ligases UBR1, UBR2, and UBR4 through the N-end rule pathway [30]. The PINK1 precursor is inserted into the mitochondrial inner membrane, where PINK1 is subjected to processing by mitochondrial proteases. The rhomboid family protease PARL, which is localized at the mitochondrial inner membrane, cleaves PINK1 at Ala103 in a $\Delta\Psi$m-dependent manner [66–70]. Cleaved PINK1 is released to the cytosol or mitochondrial intermembrane space [70]. However, the molecular mechanism of its reverse transport from the mitochondria to the cytosol remains unclear. The mitochondrial processing peptidase and hetero dimeric matrix proteases m-AAA and ClpXP are also involved in PINK1 cleavage [71]. These reports indicate that mitochondrial inner and matrix proteases coordinately regulate PINK1 stability in a $\Delta\Psi$m-dependent manner.

In *Drosophila*, loss of a PINK1-binding mitochondrial phosphatase PGAM5 improves the muscle degeneration, motor defects, and shorter lifespan caused by the loss of PINK1, suggesting that PGAM5 negatively regulates the PINK1 pathway related to mitochondrial maintenance [72]. PGAM5 knockout mice display PD-like motor dysfunction and progressive degeneration of dopaminergic neurons [73]. PGAM5S, a short form of PGAM5, recruits Drp1 and activates its GTPase activity by dephosphorylating Ser637 in Drp1, causing mitochondrial fragmentation [74]. Although the precise physiological and pathological roles of PGAM5 remain unclear, PGAM5 may play important roles in mitochondrial maintenance and PINK1-mediated mitophagy.

Lefebvre et al. identified ATPase inhibitory factor 1 (ATPIF1/IF1) as essential for Parkin translocation from cytosol to mitochondria and for mitophagy in cultured cells [75]. ATPF1

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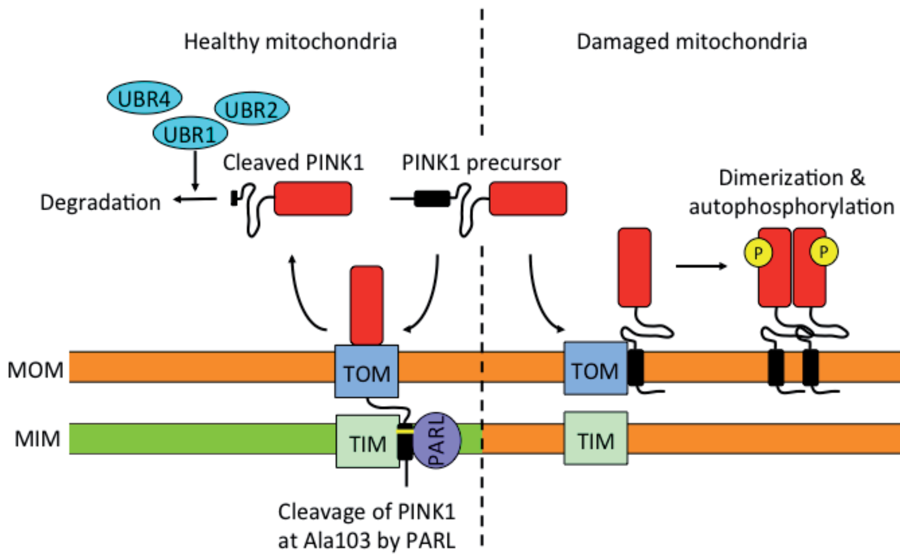


**Figure 4. Molecular dynamics of PINK1 in healthy or damaged mitochondria** In healthy mitochondria, PINK1 is imported by the TOM and TIM complexes to the mitochondrial inner membrane (MIM) and is processed by PARL, which in turn releases PINK1 into the cytosol. PINK1 is subjected to degradation by the ubiquitin ligases UBR1, UBR2, and UBR4. In damaged mitochondria, when PINK1 interacts with the TOM complex, it cannot be imported into the intermembrane space due to the loss of ΔΨm. Instead, PINK1 accumulates on the mitochondrial outer membrane (MOM), which leads to the dimerization and autophosphorylation events that activate its kinase activity.

inhibits the reversal of $F_1F_o$-ATP synthase and promotes the reduction in ΔΨm, resulting in the accumulation of PINK1 and the subsequent activation of mitophagy [75].

The master transcriptional factor for lipogenesis, sterol regulatory element binding transcription factor 1 (SREBF1), promotes Parkin translocation and mitophagy [76]. Although SREBF1 knockdown inhibits carbonyl cyanidem-chlorophenylhydrazone (CCCP)-induced PINK1 stabilization and Parkin translocation, the addition of excess amounts of cholesterol rescues these deficits, suggesting that SREBF1-dependent lipid synthesis may be a key factor in PINK1 stabilization [76].

## 5. Regulators of Parkin and mitophagy

Parkin-mediated ubiquitination of mitochondrial proteins initiates proteasomal and autophagic degradation, which involves a variety of regulators and ubiquitin- and/or LC3-binding proteins (Figure 5). The ubiquitin- and LC3-binding protein p62/SQSTM1 is required for Parkin-induced clustering of depolarized mitochondria to the perinuclear region [33, 77, 78]. Neighbor of BRCA1 gene 1 (NBR1), a functional homolog of p62, is also a ubiquitin-binding protein and is recruited to depolarized mitochondria in a PINK1-Parkin-dependent manner [79]. p62 and NBR1 expression levels coordinately change, suggesting a positive mutual regulatory relationship between p62 and NBR1 at least during viral infection [80]. Optineurin,

an autophagy receptor that binds to ubiquitinated mitochondria via ubiquitin-binding domains, is translocated to damaged mitochondria in a Parkin-dependent manner and recruits LC3 to induce mitochondrial degradation via autophagosomes [81]. However, optineurin and p62 are independently recruited to damaged mitochondria [81]. Tank-binding kinase 1 (TBK1) phosphorylates optineurin and p62, which enhances their binding affinity to LC3 and ubiquitin [82, 83]. Although TBK1 is activated downstream of Toll-like receptor 3 (TLR3) and the TLR4 signaling pathway, it is unknown which signaling event activates TBK1 during mitophagy [84]. Ambra1, an autophagy-promoting protein, is not required for Parkin translocation to depolarized mitochondria [85]. However, the interaction of Parkin with Ambra1 is potentiated during prolonged mitochondrial depolarization, resulting in the activation of the autophagy-associated class III phosphatidylinositol 3-kinase (PI3K) complex in mitochondria and their selective autophagic clearance [85].

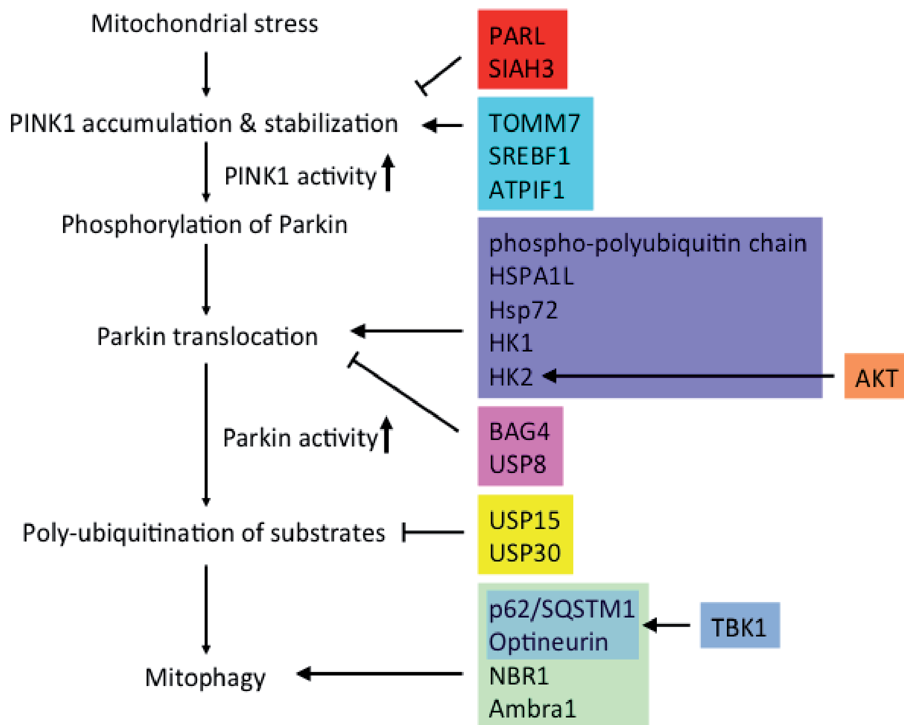


**Figure 5. Regulators of the PINK1-Parkin pathway** Various regulators of the PINK1-Parkin pathway have been characterized. ATPIF1, TOMM7, PARL, SIAH3, and SREBF1 are involved in PINK1 accumulation and stabilization on mitochondria. Mitochondrial phospho-polyubiquitin chain, HSPA1L, Hsp72, HK1, and HK2 promote Parkin translocation to mitochondria. HK2 activity is positively regulated by AKT signaling. By contrast, BAG4 and USP8 suppress Parkin translocation. The deubiquitinating enzymes USP15 and USP30 remove polyubiquitin from mitochondrial substrates of Parkin. The LC3- and ubiquitin-binding proteins p62/SQSTM1, NBR1, and optineurin link polyubiquitin on mitochondria to the autophagosome. TBK1 positively regulates the activities of p62/SQSTM1 and optineurin through phosphorylation. Ambra1 promotes autophagic clearance by activating the autophagy-associated class III phosphoinositide 3-kinase (PI3K) complex.

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Recent genome-wide RNAi screens have also identified several regulators of PINK1 and Parkin. Hasson et al. found that TOMM7, one of the TOM components, is necessary for the accumulation of PINK1 and Parkin on damaged mitochondria and chaperone proteins, HSPA1L and BAG4, have mutually opposing roles in Parkin translocation [86]. TOMM7 knockout causes impaired PINK1 import into mitochondria by inhibiting the interaction of PINK1 with the TOM complex. Moreover, a mitochondrial E3 ligase SIAH3 inhibits PINK1 accumulation probably through ubiquitination-proteasome-dependent degradation, resulting in decreased Parkin translocation [86]. Another chaperone protein, Hsp72, rapidly translocates to depolarized mitochondria prior to Parkin recruitment and interacts with both Parkin and Mfn2 only after specific mitochondrial insults [87]. Myotubes in both Hsp72 knockout mice and Parkin knockout mice exhibit increased insulin resistance and reduced maximal respiration [87]. Furthermore, myotubes in Hsp72 knockout mice exhibit impaired CCCP-induced Mfn2 degradation and Parkin-mediated LC3-II accumulation, suggesting that Hsp72 is a positive regulator of Parkin in mitophagy [87].

McCoy et al. revealed that knockdown of hexokinase (HK)1 and HK2 inhibits Parkin translocation from the cytosol to the mitochondria [88]. Inhibition of AKT signaling attenuates Parkin recruitment to mitochondria and suppresses the translocation of HK2 to mitochondria, suggesting that AKT promotes Parkin relocation [88].

## 6. Mitochondrial motility regulated by PINK1-Parkin

PINK1 and Parkin regulate mitochondrial motility in addition to mitophagy, which appears to be particularly important for neuronal function. In neurons, mitochondria are transported from the cell body to nerve terminals. Mitochondrial Rho GTPase 1 (Miro1) regulates the microtubule-dependent transport of mitochondria along with Milton, kinesin, and dynein [89]. In *Drosophila*, knockdown of PINK1 or overexpression of Miro increases the mitochondrial length in larval motor neurons and the density within nerve terminals at larval neuromuscular junctions [90]. Similar to *Drosophila*, PINK1 or Parkin overexpression suppresses both retrograde and anterograde transport of mitochondria via Miro1 degradation in rat hippocampal axons [91]. Parkin Ser65 phosphorylation by PINK1 stimulates Lys27-linked polyubiquitination of Miro1 by Parkin [40, 92]. Mfn2, a Parkin substrate, is also involved in mitochondrial transport through binding to the Miro–Milton complex on mitochondria [93]. PINK1-Parkin is thought to keep damaged mitochondria away from nerve terminals by destroying the Miro–Milton complex, thereby facilitating the removal of mitochondria in the soma via mitophagy.

## 7. Mitochondria and PD

Respiratory complex I or NADH dehydrogenase activity is significantly reduced in the substantia nigra of PD patients [94–96], implying that selective dysregulation of complex I activity is a key component of PD pathogenesis. The fact that neurotoxin 1-methyl-4-phenylpyridinium ($MPP^+$) causes selective loss of dopaminergic neurons, which causes symptoms similar to Parkinsonism, reinforces this idea [97]. $MPP^+$ is produced by the monoamine oxidase (MAO)-

mediated oxidation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and is imported into dopaminergic neurons via dopamine reuptake systems [98, 99]. In dopaminergic neurons, $MPP^+$ is concentrated in the mitochondrial matrix and binds to NADH dehydrogenase, resulting in the inhibition of OXPHOS, ATP depletion, and dopaminergic neuron death [100].

In addition to PINK1 and Parkin, other genes that have been implicated in PD are also related to mitochondrial homeostasis. Mutations in DJ-1 are associated with early-onset PD [101, 102]. DJ-1 alleviates oxidative stress through its antioxidant activity and functions as a redox-sensitive molecular chaperone [103]. Loss of DJ-1 leads to abnormal mitochondrial phenotypes, including reduced $\Delta\Psi m$, increased fragmentation and accumulation of autophagic markers [104]. CHCHD2 was isolated as a novel PD-associated gene [105] and is thought to regulate OXPHOS in mitochondria [106]. PINK1 inhibition causes decreased dopaminergic neuron viability in *Drosophila* [13, 42] and the loss of Parkin causes dopaminergic neuron-specific mitochondrial dysfunction [107]. These reports suggest that PINK1 and Parkin play critical roles in mitochondrial maintenance and dopaminergic neuron survival. Dopaminergic neurons as well as other neurons and glia cells express MAOs, which are substrates of Parkin [47, 60, 108]. During the oxidization of cytosolic or vesicular dopamine by MAOs, ROS are generated. Thus, dysregulation of MAO levels in dopaminergic neurons may account for the vulnerability of dopaminergic neurons to oxidative stress and the selective degeneration of dopaminergic neurons.

## 8. Conclusions

Two genes implicated in PD, *PINK1* and *Parkin*, are involved in the clearance of damaged mitochondria. DJ-1 and CHCHD2, which are the other gene products associated with PD, are also involved in mitochondrial homeostasis. Accumulating evidence suggests that these PD-associated genes have multifaceted roles in mitochondria, including the regulation of mitochondrial motility and quality as well as redox and respiration regulation. Although the physiological and pathological significance of newly identified phosphorylated polyubiquitin chains in PD needs to be characterized further, a complete understanding of the PINK1-Parkin pathway and its modification via therapeutic intervention would provide an opportunity to overcome a variety of mitochondrial diseases as well as PD.

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Chapter 7

# The Role of Cell Autophagy in Cancer and Its Application in Drug Discovery

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Additional information is available at the end of the chapter

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**Abstract**

Autophagy is a vital basic phenomenon that widely exists in eukaryotic cells. As one type of programmed cell death, autophagy has gained much more attention in the past few years. Recent studies suggest that the alterations in autophagy are associated with the genesis and development of cancers. It can affect cell apoptosis, angiogenesis, and treatment of tumor. Others' and our studies have found that some herbal medicines can induce autophagic cell death in cancer cell models. As herbal medicines are very important recourses for drug discovery and lead compounds of anticancer drugs, we have summarized the role of autophagy in inhibitive effect of natural products in cancer cell growth and metastasis. Finally, we present summary and critical comments on problems in current autophagy study and its future prospect.

**Keywords:** Autophagy, cancer, autophagic cell death, drug discovery, natural products

## 1. Introduction

Autophagy is a highly conserved cellular process that widely exists in eukaryotic cells. As one type of programmed cell death autophagy has gained great attention in the past few years. Recent studies suggest that autophagy is associated with the tumoriogenesis and progression of cancers. It can affect tumour cell apoptosis, angiogenesis as well as the treatment outcome of chemotherapeutics. In this chapter, we have summarized the up-to-date research about cell autophagy, including but not limited to the molecular mechanisms underlying autophagy initiation and the potential targets that autophagy regulates. As many studies have demonstrated that autophagy plays an important role in cancer initiation and progression, we also critically reviewed the double-edged sword effect of autophagy in cancer, which is reflected as the cytoprotective autophagy and cytotoxic autophagy. The role of

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autophagy in treatment and resistance of several present conventional anticancer agents is also discussed in our chapter. As Chinese medicines have a long history in treating cancer in Asian countries, we also introduced some anticancer natural products from Chinese medicines that target on autophagy in this chapter.

### 1.1. Cell autophagy overview through the up-to-date research information

Autophagy means "self-eating" in cells. It is a highly conserved cellular process among eukaryotes by which long-lived proteins and damaged organelles are packaged in the double-membrane autophagosomes and transported to lysosomes for degradation. Autophagy is one type of programmed cell death (PCD) for maintaining cell homeostasis by removing damaged or abnormal cells. It is a vital basic phenomenon that widely exists in eukaryotic cells, which has gained much more attention in the recent decades. In the process of autophagy, the intracellular damaged proteins or organelles are wrapped by the double-membrane structure autophagy vesicles and sent into the lysosome (for animal) or vacuoles (for yeast and plant) for further degradation and recycle. As autophagosome belongs to subcellular structures, normal optical microscope cannot observe it, so transmission electron microscope is needed for observing the process of autophagy. Generally, the features of phagophore are described as cup-shaped or crescent-shaped double-membrane or multilayer-membrane structure with the tendency of wrapping the cytoplasmic components. The characteristics of autophagosomes are described as double-layer or multilayer vacuole-like structure with cytoplasmic components such as mitochondria, endoplasmic reticulum, and ribosome. Autophagy lysosome is a monolayer-membrane structure in which the cytoplasmic components undergo degradation. In the study of the relationship between autophagy and cell death, the presence of a large number of autophagosomes or autophagic lysosomes were found in the cytoplasm before cell death, but these cells lack typical apoptosis features such as nuclear pyknosis and karyorhexis or cell shrinkage and the formation of apoptotic bodies. So this is a new type of programmed cell death which is different from apoptosis. In order to distinguish between these two types of cell death, autophagy is also called Type II cell death. In contrast, apoptosis is called Type I cell death. However, whether autophagy is the direct cause of cell death is still controversial. Some researchers believe that cell death is caused by the process of autophagy, while some think that autophagy is not the direct reason for cell death.

As a main intracellular degradation and recycling process, autophagy is important for maintaining energy homeostasis and cellular remodeling during physiological process. Recent studies have suggested that alterations in autophagy can affect the genesis and development in several diseases such as inflammatory disease, heart disease, neurodegenerative disease, and cancer. A common characteristic in these diseases is a dysfunction of autophagy, which influenced the susceptibility of programmed cell death. Our knowledge of the functions and regulation of this programmed cell death pathway has increased substantially in recent years from studies conducted in Drosophila and yeast, as well as in mammalian cells and tissues.

Generally, there are three types of autophagy: chaperone-mediated autophagy, microautophagy, and macroautophagy. For chaperone-mediated autophagy, single intracellular proteins are recognized by a chaperone complex that improves target-motif-governed transport,

lysosomal membrane binding, migration, and subsequent internalization. For microautophagy, it utilizes lysosomal limiting membrane invagination, septation, or protrusion to transport cytoplasmic materials. Macroautophagy is a highly conserved cellular process by which cells sequester a part of their cytoplasm or organelles into the autophagosomes that will fuse with lysosomes for degradation and recycling of the enclosed materials. Of the above three types of autophagy, most of our current studies are focused on macroautophagy (hereafter referred to as autophagy), which is also the focus of this chapter. Although all these three types of autophagy differ in the way by which cargo material is transported to the lysosome, they are similar in the last step of lysosomal protein degradation by hydrolase exposure.

Autophagy is very important for cellular homeostasis. It can promote the catabolism of denatured proteins or damaged organelles and the elimination of long-lived proteins. In addition, autophagy is an important adaptive mechanism. It is augmented obviously when the cells need to cope with certain cellular stresses such as starvation. Recent studies have demonstrated that autophagy also plays pathological roles sometimes. But the molecular mechanism of these pathological effects remains unknown.

## 2. The molecular mechanisms of autophagy

### 2.1. The initial stage of autophagy

In the initial stage, serine–threonine protein kinase ULK1 promotes the autophagy process. Autophagy inducers such as the defective nutrition or exposure to some chemotherapy agents cause dephosphorylation of ULK1; the ULK1 complex consists of ULK1, ATG13, FIP200 and receives stress signals from mTOR complex 1. When mTORC1 kinase activity is inhibited, autophagosome formation occurs. ULK1 can activate the phosphorylation of ATG13 and FIP200 to start the initial stage of autophagy.

### 2.2. Double-membrane vesicle formation stage of autophagy

In this stage, the Beclin-1–Vps34 complex, which consists of Vps34, Beclin-1, ATG14L, and Vps15, takes part in the process of vesicle nucleation. Under certain circumstances that induce autophagy, the dephosphorylated ULK1 complex musters the Beclin-1–Vps34 complex to form the autophagosome by phosphorylation of Ambra1. In this stage, TRAF6 interacts with Ambra1, which acts as an E3 ubiquitin ligase for ULK1, which results in stabilization and self-association of ULK1 by K63 ubiquitination. The dephosphorylated ULK1 complex also facilitates the activity of the ATG14L-containing Vps34 complex by phosphorylation of Beclin-1. Furthermore, EGFR and AKT regulate autophagy by phosphorylation of Beclin-1 without the impact of mTORC1. Next, the phosphatidylinositol-3-phosphate created by Vps34 musters an effector protein to facilitate the formation of double-membrane vesicle. The WIPI protein, which is the transcription product of a member of the ectopic P-granule subset of the metazoan-specific autophagy gene family, also participates in this stage.

### 2.3. Double-membrane vesicle elongation stage of autophagy

ATG proteins participate in the accomplishing of the autophagosome and fit out into two ubiquitin-like conjugation systems, ATG8 (LC3)–phosphatidylethanolamine and ATG12–ATG5–ATG16L. For the ATG12–ATG5–ATG16L system, ATG12 is connected to ATG5 by the synergistic action of ATG107 and ATG. The ATG12–ATG5–ATG16L complex consists of ATG12–ATG5 and ATG16L by conjugate binding, which takes part in the LC3–phosphatidylethanolamine conjugation. LC3, created by the ATG4 protease from Pro-LC3, is connected to phosphatidylethanolamine by ATG3, ATG7, and the ATG12–ATG5–ATG16L complex. After the above procedure, the lipid-conjugated LC3, which is positioned to the double-membrane of the autophagosomes, takes part in the formation and elongation of autophagosomes.

### 2.4. Fusion and degradation stage of autophagy

The captured proteins or organelles waiting for degradation in an engulfing or developing autophagosome are promoted by autophagy adaptor proteins, or receptors act as a connection. The complete autophagosomes will fuse with lysosomes to become autolysosomes. In these structures, the captured organelles and materials will be digested by lysosomal enzymes.

### 2.5. The final stage of autophagy

In the final stage, an essential component of the mTORC1 complex (mTOR) is reactivated by nutrients created by the autolysosomes. This process is very important for depressing the redundant activation of autophagy when the cells are in the periods of starvation. The mTOR generated from this stage can produce raw materials to form new lysosomes so that the lysosomes do not reduce during the autophagy procedure.

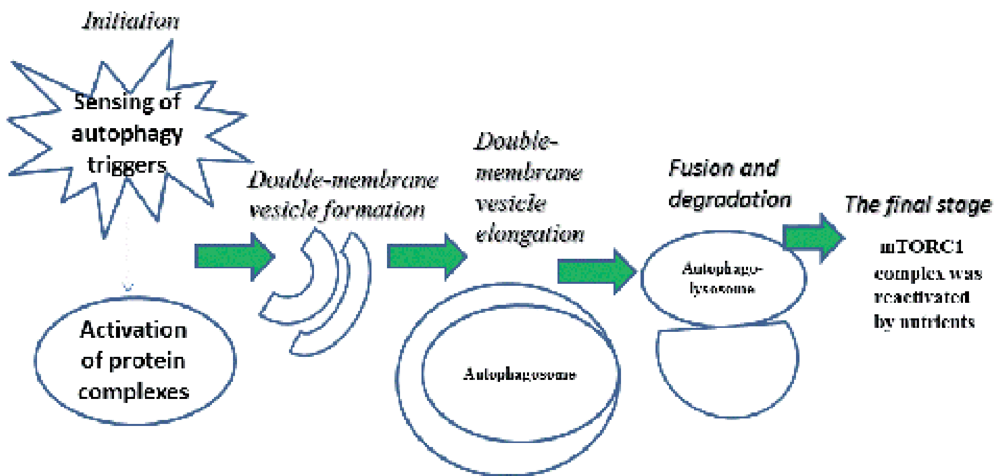



**Figure 1.** The process of autophagy

## 3. Major autophagy regulation target

cAMP-dependent protein kinases A (PKA), mammalian target of rapamycin complex 1 (MTORC1), and AMP-activated protein kinase (AMPK) are three major kinases for regulating cell autophagy. These kinases, along with some other molecules such as CAMKK2/CaMKKβ and TSC1/2, participate in a wide range of intracellular or extracellular signal pathways to regulate autophagy.

### 3.1. cAMP-dependent Protein Kinase A (PKA)

When the cell faces some stresses such as absence of growth factors, nutrient starvation, hypoxia, or endoplasmic reticulum stress, the survival of these cells can be dependent on autophagy. Recent research has shown that Ras/PKA (cAMP-dependent protein kinase) signaling pathway was associated with the early stage of autophagy in yeast. The studies showed that Ras/PKA signaling pathway can suppress the early stage of autophagy in *Saccharomyces cerevisiae* cells when faced with nutrient-rich conditions [20]. In mammals, this suppression is conducted at least partially by the phosphorylation of microtubule-associate protein 1 light chain 3 by PKA.

### 3.2. Mammalian Target of Rapamycin Complex 1 (MTORC1)

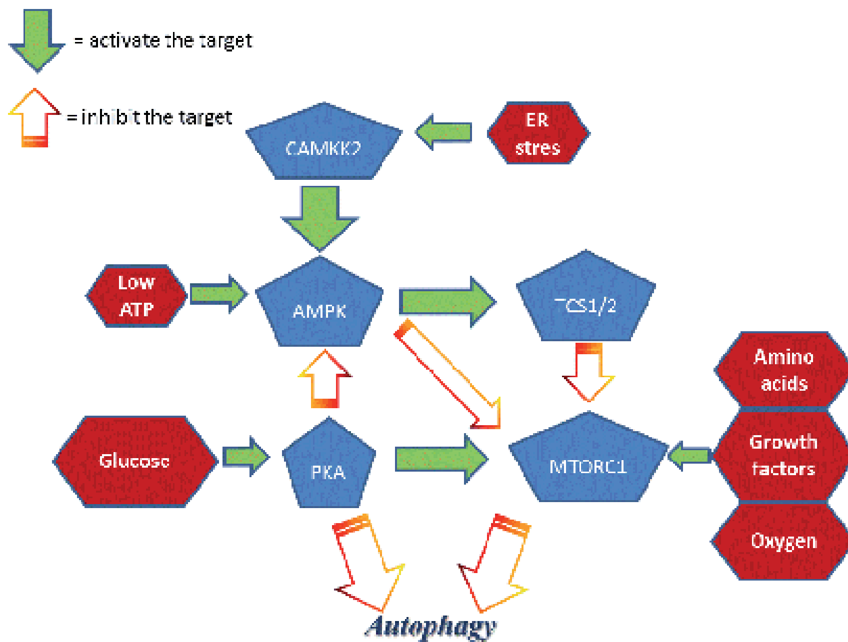
Studies have shown that MTORC1 is activated by the presence of amino acids. Amino acids can activate RAG (RAS-GTPases) proteins that regulate MTORC1. Recent research has demonstrated that in *Drosophila melanogaster* S2 cells, knockdown of Rag gene inhibited the activation effect of amino acids on TORC1. The constitutively active (GTP-bound) Rag gene expression can activate TORC1 without the presence of amino acids signals while expression of dominant-negative Rag can inhibit the activation effects of amino acids signals on TORC1. Researchers believe that there is some cross talk between these pathways. For example, PKA can directly activate MTORC1 and also indirectly activate MTORC1 by suppression of the AMPK.

### 3.3. AMP-activated protein Kinase (AMPK)

AMPK can respond to the change of ATP/AMP levels in cells and act as an intracellular ATP/AMP-sensing kinase. It is also a substrate of PKA and participates in regulating autophagy. AMP can activate the activity of AMPK, while ATP binding suppresses this kinase activity. AMPK can phosphorylate the TSC1/2 complex when activated by low ATP/AMP levels. Then, the phosphorylated TSC1/2 complex can indirectly or directly suppress the activity of MTORC1. Several studies have also demonstrated that AMPK can induce autophagy by activating ULK1.

### 3.4. Other regulating mechanisms

Some studies have proved that ER stress can cause autophagy by leading to $Ca^{2+}$ concentrations' upregulation and inducing calcium/calmodulin-dependent protein kinase 2, beta




(Modified from: Antioxid Redox Signal. 2014 Jan 20; 20(3): 460–473)

**Figure 2.** The regulation mechanisms of autophagy

(CAMKK2/CaMKKβ), to activate AMPK [29]. (UPR) signaling also plays an important role in ER stress-related autophagy. This signal pathway has been proved in both mammals and yeast models. However, the final results of autophagy in response to ER stress are still not clear. Some researchers insist that it will improve cell survival while others believe that it may lead to cell death.

## 4. The relationship of cell autophagy and cancer

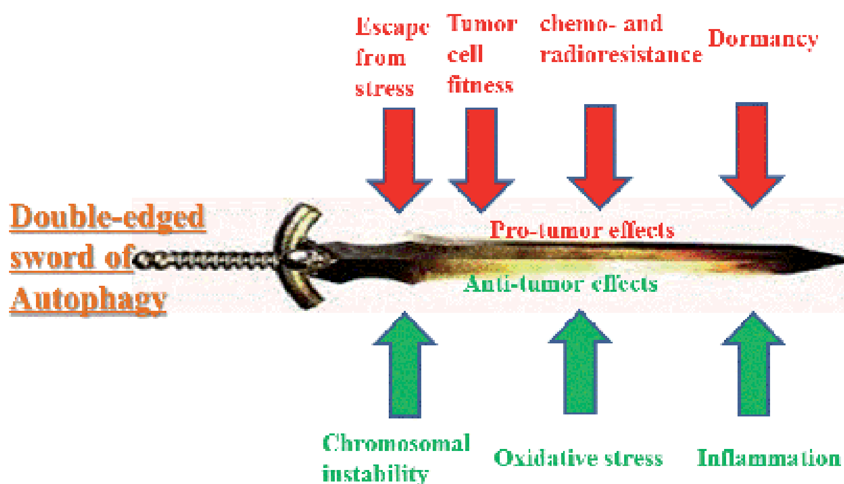
### 4.1. Autophagy: A double-edged sword in cancer

Recent studies have demonstrated that autophagy plays an important role in cancer initiation and progression stages. In some studies, autophagy has shown tumor inhibition effect by inducing autophagic cell death. But some studies have also proved that autophagy can facilitate cell survival during cancer treatment or other stresses. Many cancer researchers have recognized that autophagy is a double-edged sword in cancer. Usually, the role of autophagy in cancer depends on the tumor stage, the tumor genotype and microenvironment, as well as the type of therapy. For example, some recent studies have found that different kinds of autophagy-inducing drugs can play different roles in cancer treatment. In one study, the researchers used TRAIL and Fas ligand to treat cell lines with different autophagic levels

respectively. These two compounds can both activate the cell death receptors and induce programmed cell death.

## 4.2. The cytoprotective autophagy in cancer

Previous studies have confirmed that autophagy can lead to resistance against metabolic stress and protect tumor cells through recycling organelles or proteins. In a study of Kirsten rat model, the sarcoma-activating viral oncogene homolog (K-Ras) variations can upregulate autophagy and facilitate tumor cell survival by autophagy. Some studies also demonstrated that both radiation therapy and chemotherapeutic agents can lead to autophagy and facilitate tumor cell survival. Although these studies have proved that the conventional cancer therapy may induce autophagy and reduce tumor cell apoptosis, no direct evidence has proved that inhibition of cell autophagy can increase the sensitivity of chemotherapy and radiation for cancer treatment. So the cytoprotective role by autophagy in cancer still needs more supportive evidence. If we can prove this cytoprotective form of autophagy in cancer, we can reduce the therapy resistance by inhibiting tumor cell autophagy during conventional cancer treatment [37].




(Modified from: Semin Cancer Biol. 2013 Aug;23(4):252-61.)

**Figure 3.** Autophagy: a double-edged sword in cancer

## 4.3. The cytotoxic and cancer inhibitory autophagy

As we have discussed above, many studies have shown the cytotoxic form of autophagy in cancer. One study has combined vitamin D with radiation therapy for breast tumor model. The results showed that autophagy induced by the therapy can facilitate cell death in breast tumor. According to recent studies on autophagy, the cytotoxic form of autophagy was generated on directly killing cells by itself or further inducing apoptosis-related cell death. Some recent studies have also discovered that the autophagy-related genes such as Beclin 1

can suppress tumor devolvement in both human and mice cells. Further studies demonstrated that this tumor suppression effect by autophagy was related to the Akt pathway. Some antitumor agents also have been established with the activity of inducing autophagy-related cell death and tumor suppression [39]. Autophagy deficiency can induce the accumulation of the autophagy substrate P62 and promote the tumor growth through the NF-kB pathway. The deficiency of autophagy-related genes such as Beclin-1, UVRAG, and Bif-1 has been confirmed in many different tumor types. These findings further strengthen the potential linking between autophagy and cancer inhibition. Autophagy-related cell death and tumor suppression are very important in recent anticancer research especially when tumor cells are deficient in essential apoptotic modulators such as caspases and Bcl-2 family. The cytotoxic and cancer inhibitory autophagy may be a potential target for developing novel cancer therapy, although there are still some unsolved problems in this field. In future studies, the molecular mechanisms of cytotoxic and cancer inhibitory autophagy still need further exploration.

## 5. Anticancer drug discovery target on autophagy

### 5.1. Overview of conventional anticancer agent target on autophagy

One of the characteristics of tumor cells is their capacity to escape from apoptosis. Recent studies have suggested that in some conditions, nonapoptotic cell death can occur in tumor cells, which may related with autophagy. Although there are few antitumor drugs that target autophagy in present clinical cancer therapy, many existing antitumor drugs can suppress tumor growth with more or less connections with autophagy. Some drugs can induce autophagic cell death directly or indirectly by autophagic apoptosis or necrosis. Recent research has shown that alkali, vincaleukoblastinum, arsenic trioxide, rapamycin, and vitamin D derivatives can induce autophagic cell death in some cancer cells; while LAK (lymphokine activated killer cell) can induce autophagic cell death in T98G and U373MG glioblastoma cell lines 24–96 hours after the treatment. On the other hand, autophagy can also play a tumor cell protective role by promoting intracellular material cycle and isolating harmful substances. For example, conventional anticancer drugs, such as some hormonal agents, and chemotherapy drugs may induce treatment resistance by autophagy. In this case, combination use of autophagy inhibitor with these drugs is important for clinical therapy. Recent studies have found that in particular microenvironment or genetic backgrounds, autophagy can play cytotoxic or tumor inhibitory roles. Therefore, context-specific autophagy regulations can be a promising novel therapeutic attempt to facilitate the present available antitumor therapy. Representative autophagy inducers or inhibitors designed for the purposes of promoting or inhibiting cancer progression are described in Table 1. Among the present studied autophagy-based anticancer drugs, some autophagy-modulating agents such as and rapamycin have been approved for clinical use in cancer management ; thus, autophagy has been established as a promising therapeutic target in cancer treatment.

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### 5.2. Anticancer natural products from Chinese medicines target on autophagy

Chinese medicines have a long history in treating cancer in some Asian countries and districts. It is one of the most popular alternative and complementary medicines used for cancer therapy. The effect of Chinese medicines on survival and improving the quality of life has been identified by many basic and clinical studies. Recent research has proved that some of these natural products from Chinese medicines can induce autophagy in tumor cells, while some of them can suppress autophagy in cancer. Y. Lao et al. found that oblongifolin C, which is isolated from the herb *Garcinia yunnanensis* Hu can improve the antitumor efficiency of nutrient deprivation by inhibiting autophagy. Normally, cancer cells can resist nutrient deprivation by inducing autophagy and escaping from cell death, but oblongifolin C can inhibit autophagy in this circumstance and thus exhibit antitumor effect. M. Chen et al. reported that Ophiopogonin B, which is isolated from the herb Radix *Ophiopogon Japonicus,* can suppress cell cycle, inducing autophagic cell death in lung cancer cells in vitro. M. Pedro et al. and their research teams found that seven natural prenylated flavones extracted from Chinese medicine herbs can induce autophagy in MCF-7 cells and suppress tumor cell growth. Honokiol, which is isolated from the herb *Mangnolia officinalis,* has been demonstrated with anticancer effect by inducing autophagic cell death on melanoma cells; further study of mechanisms has confirmed that the related signal pathways are Notch signaling and AKT/mammalian target of rapamycin. The herb *Syzygium samarangense's* active compound dimethyl cardamonin can inhibit the growth of colorectal carcinoma HCT116 cells by inducing autophagy; the molecular mechanism study confirmed that the microtubule-associated protein (LC3)-I-LC3-II is related with the autophagic cell death by this agent. Law reported that the natural product from herb *Alisma orientale* can induce autophagy and cell cycle arrest in cancer cells through clearing cellular stress; this discovery has been further proved by another research team that also used a Chinese medicine active compound cucurbitacin B to induce cell autophagy. Research from our lab in The University of Hong Kong demonstrated that fangchinoline (isolated from Fangji, *Stephenia tetrandra* S Moore) can induce autophagic cell death in two human hepatocellular carcinoma cell lines HepG2 and PLC/PRF/5. Further descriptions of the mechanisms of some isolates from Chinese medicines targeting on autophagy are presented in Table 1. These natural products from Chinese medicines are very important sources for cancer treatment, but it still needs more molecular mechanism studies in the future.

| Agent | Target | Regulation mechanisms | Tumor type | Role in cancer |
|---|---|---|---|---|
| Rapamycin | mTORC1 | mTORC1 inhibitors | Malignant glioma | pro-death |
| Berberine* | | | hepatocellular carcinoma | pro-death |
| aArsenic trioxide* | | | Melanoma<br>Ovarian cancer | pro-death |
| uUrsolic acid* | | | Breast cancer | pro-death |
| INNO-406 | BCR-ABL | Tyrosine kinase inhibitors | Chronic myeloid leukemia | pro-death |

| Agent | Target | Regulation mechanisms | Tumor type | Role in cancer |
|---|---|---|---|---|
| | | | Gastrointestinal stromal tumor | pro-survival |
| Imatinib | | | Chronic myeloid leukemia | pro-survival |
| Dasatinib | SRC/ABL | Tyrosine kinase inhibitors | Ovarian cancer | pro-death |
| | | | Chronic lymphocytic leukemia | pro-survival |
| Baicalin* | Akt | Akt inhibitors | Bladder cancer cells | pro-survival |
| Honokiol * | | | Malignant melanoma cells | pro-death |
| Fangchinoline* | AMPK | AMPK activators | Hepatocellular carcinoma | pro-death |
| NPI-0052 | Proteasome | Proteasome inhibitors | Ovarian cancer | pro-survival |
| | | | Prostate cancer | pro- death |
| Bortezomib | | | Prostate cancer | pro-survival |
| Vorinostat | Histone deacetylases | Histone deacetylases inhibitors | Gastric cancer | pro-survival |
| SAHA | | | Chondrosarcoma, endometrial stromal sarcoma, hepatocellular carcinoma | pro-death |
| Pseudolaric acid B* | BH3 domain | Bcl-2 inhibitors | Prostate cancer | pro-death |
| Oridonin* | | | Malignant glioma | pro-survival |
| Z18 | | | Breast cancer | pro-death |
| Oridonin* | NF-κB | NF-κB inhibitors | Colon cancer | pro-survival |
| Silibinin* | NF-κB | NF-κB activators | Multiple cancer | pro-death |
| Etoposide* | Topoisomerase II | | Hepatocellular carcinoma | pro-death |
| 3-MA | PI3K | PI3K inhibitor | Colon cancer, esophageal squamous cell carcinoma | pro-survival |
| CQ | Lysosome | Lysosomotropic agents | Glioblastoma, colon cancer | pro-survival |
| HCQ | | | Breast cancer | pro-survival |
| Dimethyl cardamonin* | (LC3)-I-LC3-II | (LC3)-I-LC3-II activator | Colorectal carcinoma cells | pro-death |

* represents natural products from Chinese medicines

(Modified from: *Acta Pharmacol Sin. 2013 May; 34(5): 612–624*)

**Table 1.** Representative antitumor agents target on autophagy

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## 6. Summary: Problems in current autophagy study in cancer and future prospects

Autophagy is a homeostatic process that is highly conserved across different types of mammalian cells. Sustained and excessive autophagy may lead to cell death and tumor shrinkage. Autophagy is able to relieve tumor cell from oxidative and nutrient stress during the rapid expansion of cancer. The mechanisms and the role of autophagy in human cancer are complicated. Many recent discoveries in basic research have found that targeting autophagy may be a potential novel therapeutic solution for treating cancer. Technical variations in detecting autophagy affect data quality, and studies should focus on elaborating the role of autophagy in deciding cell fate. Although our knowledge on autophagy in cancer research has increased rapidly in the last few decades, there are still some problems in the current study of autophagy. How to monitor a dynamic autophagy process with improved technique and method? Why does autophagy have either tumor protective or inhibitor role at different cancer stages and in different microenvironments? How does autophagy regulate varies of tumor generation or inhibition pathways? Would targeting the autophagy-related cancer pathways be a novel strategy for anticancer therapy? Also, as cancer is a systemic disease, many autophagy-related cross-talk pathways need to be further studied at a systems level in future research. According to previous studies, some chemical agents have been proved with the ability of inducing or suppressing autophagy in vitro, but few in vivo data are available in this field. More in vivo studies on detecting autophagy in tumor initiation and development are urgently required for drugs development in current cancer research and wide evidence-based clinical research on the relationship between autophagy and therapy efficacy are also necessary in future studies. It was shown in the literature that many anticancer natural compounds and extracts could initiate autophagy in tumor cells as summarized in this chapter. Natural-products-induced autophagy could protect tumor cells from apoptotic death in some cases. This is an emerging issue, and it is necessary to study the role of autophagy in tumor suppressive effect of natural products.

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Chapter 8

# Modulation of Autophagy by Free Fatty Acids

Jiuhui Wang and Daotai Nie

Additional information is available at the end of the chapter

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**Abstract**

Fatty acids are important molecules with multiple biological properties. Emerging evidence suggests that fatty acids can modulate autophagy. Saturated fatty acids contribute to pancreatic β-cell dysfunction in type 2 diabetes. Palmitic acid, one of the long-chain saturated fatty acids (LCFA), induces autophagy of β-cells which protects them from dysfunctions and apoptotic cell death. Short-chain fatty acids (SCFA) possess antitumor activity in colon cancer cells by promoting autophagy. SCFAs can induce autophagy by suppressing the activity of mTOR signaling. As the most common monosaturated fatty acid (MUFA) in daily nutrition, oleic acid could induce autophagy, which is responsible for the regulation of lipids metabolism in hepatocytes. The ω-3 and ω-6 polyunsaturated fatty acids (PUFA) are essential in normal physiology and metabolism and play a contributory role in the incidence and progress of a series of disease including cancer. Autophagy triggered by ω-3 PUFAs contributes to the cytotoxicity in cancer cells by enhancing apoptosis, while autophagy mediated by ω-6 PUFAs led to the increase in *Caenorhabditis elegans* lifespan. The recent findings illustrate the potential involvement of autophagy regulation by fatty acids in a number of biological and pathological processes.

**Keywords:** Autophagy, fatty acids, lipids, apoptosis, cancer

## 1. Introduction

Fatty acids are aliphatic carboxylic acids consisting of a hydrocarbon chain and a terminal carboxyl group. Fatty acids are classified as several groups with respect to their structure and biological functions. Saturated fatty acids, which have no double bond between individual carbon atoms of the hydrocarbon chain, are divided into short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA), long-chain fatty acids (LCFA), and very long-chain fatty

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acids (VLCFA) according to their chain length. The introduction of double bonds in the hydrocarbon chain results in the formation of the unsaturated fatty acids. Monounsaturated fatty acids (MUFA) have only one carbon–carbon double bond which can occur in different positions. Polyunsaturated fatty acids (PUFA) contain two or more double bonds along their carbon backbones. PUFAs are classified into subgroups including ω-3 and ω-6 PUFAs according to the position of the first double bond starting from methyl end.

Fatty acids provided by diets are ingested as triglycerides and then broken down into free fatty acids and monoglycerides for absorption in small intestine. In the intestinal mucosa cells, free fatty acids are re-esterified to triglycerides which are transported via lymphatic vessels to circulation as part of chylomicrons. Eventually, they bind to the membranes of hepatocytes, adipocytes, or muscle fibers where they are either stored or oxidized for supplying energy. Apart from serving as important energy substrates, fatty acids have multiple biological functions. The ω-3 and ω-6 PUFAs are essential in normal physiology and metabolism through their roles in membrane structural lipids, signal transduction pathways, eicosanoids metabolism, and gene expression. Saturated fatty acids have been related to adverse health effects whereas unsaturated fatty acids, especially monounsaturated and ω-3 polyunsaturated, are thought to be protective. In addition, trans fatty acids have been shown to play a negative role on health whereas conjugated fatty acids might be beneficial [1].

Autophagy is a catabolic process in which cytoplasmic components are sequestered in double-membrane vesicles and degraded by fusion with lysosomal compartments. The process of autophagy begins with the formation and elongation of phagophores. The cytoplasmic cargo is then sequestered and the double-membrane autophagosome fuses with a lysosome to generate the autolysosome. Finally, degradation is achieved through the hydrolytic enzymes within the autolysosome [2]. Autophagy maintains cellular homeostasis by eliminating superfluous or damaged proteins and organelles as well as provides substrates for energy generation and biosynthesis. Deregulation of autophagy is linked with susceptibility to various disorders including degenerative diseases, metabolic syndrome, aging, infectious diseases, and tumorigenesis.

Emerging evidence indicates the ability of fatty acids to induce autophagy. Mice fed with a high-fat diet lead to the formation of double-membrane autophagosomes in livers and increase the level of LC3-II which serve as a marker of autophagosome formation and the activity of autophagic flux [3]. Autophagy has been demonstrated to be involved in the biological function of fatty acids. Our recent studies reported that SCFAs promote autophagy in colon cancer cells which serves as an adaptive strategy for retarding apoptotic cell death [4]. In this chapter, the regulation of autophagy by fatty acids will be discussed including the mechanism of action involved and the biological significance implicated.

## 2. Saturated fatty acids

Saturated fatty acids are saturated with hydrogen and can be divided into subgroups according to their chain length. LCFAs increase levels of low-density lipoprotein-cholesterol, which is

connected with pathogenesis in multiple organs. SCFAs influence a wide array of cellular functions, especially affecting colonic health. Autophagy triggered by LCFAs in pancreatic β-cell and cardiomyocytes plays a critical role in the molecular pathogenesis of lipotoxicity. SCFAs induce autophagy in colon cancer and protect against mitochondria-mediated apoptotic cell death.

### 2.1. Long-chain saturated fatty acids

#### *2.1.1. Palmitic acid*

Palmitic acid (16:0) is the most abundant free saturated fatty acid in bloodstream. As its name indicates, it is a major component of the palm oil but also be found in meats and diary products. The stimulatory effect of palmitic acid on autophagy has been extensively examined in rat pancreatic beta cell INS-1 and has also been observed in human pancreatic islet and many other human cell lines including in neuroblastoma, myoblasts, hepatocytes, and endothelial cells [5-7]. After exposure to palmitic acid, INS-1 cells displayed a marked increase in the number of typical autophagosomes characterized by double-membranous vacuoles engulfing cytoplasmic structures. When monodansylcadaverine (MDC) was used to label autophagic vacuoles, the proportion of cells with MDC-stained dots dramatically increased after palmitic acid treatment. In GFP-LC3-expressed INS-1 cells, the number of GFP-LC3 puncta began to increase from 6 h after palmitic acid treatment and size of the dots gradually increased during palmitic acid incubation [5]. Autophagic flux was also accelerated by palmitic acid as evidenced by the enhanced conversion of LC3-I to LC3-II and the fusion between autophagosome and lysosomes. During autophagy, long-lived proteins were engulfed by autophagosomes and delivered to the lysosomes for degradation. The degradation rate of long-lived protein significantly increased in palmitate-treated cells. When autophagy was suppressed by ATG7 knockdown with siRNA, the proteolytic rate induced by palmitic acid significantly declined [6].

While mammalian target of rapamycin (mTOR) has been well established as the key regulator of the autophagic process, palmitic acid does not change phosphorylated status level of mTOR or its substrate p70S6K, suggesting that autophagy triggered by palmitic acid is mTOR-independent [8]. c-Jun N-terminal kinases (JNK) have been implicated in the induction of autophagy by various stimuli including starvation, cytokine stimulation, and endoplasmic reticulum (ER) stress. One mechanism by which JNK contributes to autophagy involves phosphorylation of the antiapoptotic protein Bcl-2. Phosphorylation of Bcl-2 by JNK causes its dissociation from Beclin-1 resulting in induction of autophagy. In addition, JNK could regulate autophagy by enhancing the expression of autophagic genes including Beclin-1, ATG5, and ATG7 [9]. JNK1 was phosphorylated shortly after exposure to palmitate in INS-1 cells, and inhibition of JNK with chemical inhibitor or molecular methods significantly decreased palmitate-induced conversion of LC3-I to LC3-II, indicating that JNK1 activation is responsible for palmitic-acid-triggered autophagy [6,10].

Another kinase involved in palmitate-induced autophagy is protein kinase C (PKC). There are 10 PKC isoenzymes classified as conventional, novel, and atypical in mammalian system. Several PKC isoenzymes including PCKδ, PKCθ, and PCK$\alpha$ have been implicated in autophagy. When taken up by cells, excess free fatty acids are converted into acyl-coA derivatives which can be incorporated and stored in the cells as neutral lipids like diacylglycerol (DAG) and triacylglycerol (TAG). Treatment with palmitic acid resulted in an increase in the relative levels of DAG, which serves as a natural agonist to recruit PKC proteins to plasma membrane for activation [11]. Conventional PKC$\alpha$ has been demonstrated to be activated and responsible for the autophagy by palmitic acid as evidenced by the following observations:

1. The formation of GFP-LC3 puncta and the conversion of LC3-I to LC3-II induced by palmitic acid could be significantly reduced by the chemical inhibitors of general and conventional PKCs.,
2. There was enhanced phosphorylated PKC$\alpha$ in palmitic-acid-treated cells compared to the control group. and/or
3. siRNA knock-down of PKC$\alpha$ reduced the level of LC3-II and autophagic flux induced by palmitic acid [8].

Lipotoxicity is a metabolic syndrome that excessive accumulation of lipid in non-adipose cells leads to cellular dysfunctions and death. Lipotoxicity is believed to be critically involved in type 2 diabetes mellitus which is characterized with the decline of β-cell and insulin resistance. Chronic exposure to elevated levels of free fatty acids leads to pancreatic β-cells lipid overload, dysregulation of insulin secretion, and apoptotic cell death [12]. The lipotoxicity of palmitic acid was predominant when cells were exposed to high level of saturated fatty acids. However, palmitic-acid-mediated autophagy induction has been suggested to be a protective mechanism against free fatty acids-induced β-cell dysfunction, apoptotic cell death, and insulin resistance [5,13-14]. Therefore, autophagy plays a crucial role in the preservation of pancreatic β-cell function. Further, altered autophagic activity has been implicated in the progression of obesity to type 2 diabetes through impairment of β-cell functions and development of insulin resistance [15]. Blockage of mTOR signaling pathway with rapamycin increases the formation of autophagosomes but attenuates palmitic-acid-induced β-cells death. On the contrary, reduction of autophagosome formation by knocking down the ATG5, inhibition of fusion between autophagosome and lysosome by bafilomycin A1, or inhibition of proteolytic degradation with E64d/pepstatin A could significantly augment palmitic-acid-induced β-cells death [5].

*2.1.2. Myristic acid*

Myristic acid (14:0) accounts for small amounts of total fatty acids in animal tissue, but is more abundant in milk fat and copra and palmist oils. Like other dietary saturated fatty acids, myristic acid is usually associated with negative consequences for human health. Russo et al. [16] found that there was high expression level of LC3 and BECN1 in whole heart lysate and increased number of LC3 puncta in left ventricles of mice fed with milk fat-based diet (MFBD). Because of the high myristate content of MFBD, the effects of myristate on autophagy in isolated mouse cardiomyocytes were examined. Treatment with

myristate promoted the overexpression of autophagy marker BECN1 which was sufficient to increase cardiac autophagy. Furthermore, myristate led to increased formation of GFP-LC3 puncta and the conversion of LC3-I to LC3-II, suggesting the ability of myristate to active autophagic flux in cardiomyocytes. In addition, treatment with palmitate did not induce expression of autophagy marker, indicating that myristate but not palmitate is responsible for the autophagy induced by MFBD [16].

The molecular mechanism underlying myristate-mediated autophagy in mouse cardiomyocytes is involved in the upregulation of C14-ceramide and ceramide synthase 5 (CerS5). Ceramide, which is composed of sphingosine and fatty acid, is a sphingolipid bioactive molecule that can trigger autophagy by interfering with the mTOR signaling pathway and dissociation of the Beclin-1:Bcl-2 complex in a JNK1-mediated Bcl-2 phosphorylation-dependent manner [17]. Myristate treatment increased C14-ceramide levels 10-folds in isolated mouse cardiomyocytes. Meanwhile, the induction of C14-ceramide was consistently observed in the heart of mice fed with MFBD. Ceramide synthesis occurs through N-acylation of sphingoid base by one of 6 CerS isoforms, and CerS5 is responsible for the C14-ceramide synthesis in the mouse heart. Gain- and loss-of-function experiments demonstrated the requirement of CerS5 in sphingolipid-induced autophagy in cardiomyocytes. Overexpression of CerS5 induced BECN1 expression in isolated cardiomyocytes even in the absence of myristate treatment. siRNA-mediated knockdown of endogenous CerS5 abrogated the induction of BECN1 expression, the formation of GFP-LC3 puncta, and the conversion of LC3-I to LC3-II in isolated mice cardiomyocytes treated with myristate, indicating the critical role of CerS5 in myristate-triggered autophagy.

Autophagy has been identified as an important process upregulated in cardiac hypertrophy and dysfunction in responses to pressure overload. However, whether autophagy plays a beneficial or detrimental role in cardiac hypertrophy remains unclear [18]. Mice fed with MFBD developed left ventricular hypertrophy and functional reduction in ejection fraction. Myristate, but not palmitate, treatment increased the size of isolated cardiomyocytes, suggesting that myristate is responsible for MFBD-induced cardiac hypertrophy. In fact, myristate-induced hypertrophy could be completely prevented by LC3 knockdown or treatment with autophagy inhibitor 3-methyladenine (3-MA), indicating that autophagy induced by myristate plays a prohypertrophic role in cardiac lipid overload [16].

### 2.2. Short-chain fatty acids

Short-chains fatty (SCFAs) acids are the major byproducts of bacterial fermentation of undigested dietary fiber within the intestinal lumen. The main SCFA production of fiber fermentation in colon is acetate (2:0), propionate (3:0), and butyrate (4:0) in a molar ratio about 3:1:1. More than 95% of the SCFAs produced in intestine are rapidly absorbed and metabolized by the host. SCFAs exert potent effects on a variety of colonic mucosal function such as inhibition of inflammation reinforcing various components of colonic defense barrier and decreasing oxidative stress. Furthermore, SCFAs have antitumor activity in the colon by promoting apoptotic cell death [19].

We have reported that SCFAs, particularly propionate, induced autophagy characterized by increased LC3 puncta formation, enhanced conversion of LC3-I to LC3-II, and upregulated expression of LAMP-2 in colon cancer cells [4]. In addition, autophagy could be induced by butyrate in gingival epithelial cells and Chinese hamster ovary (CHO) cell, which play a potential role of cell survival mechanism [20,21].

Treatment of colon cancer HCT116 cells with propionate led to a strong time-dependent reduction in the phosphorylation state of mTOR at Ser2481 while there was no change in the total mTOR levels. Phosphorylation of eukaryotic initiation factor 4E-binding proteins (4E-BP1), a key downstream effector of mTOR activity, also markedly decreased in a dose- and time-dependent manner after exposure to propionate. Furthermore, reduced level of phosphorylated p70S6K, another key downstream effector of mTOR, was noted by 7 h following propionate treatment. Collectively, these observations demonstrate that downregulation of the mTOR signaling pathway is a mechanism for propionate to induce autophagy [4]. To further characterize the mechanisms underlying the reduced mTOR activation and concomitant autophagy induction by SCFA, we examined the activation status of PI3/Akt pathway which activates mTOR in response to the introduction of nutrient and growth factors, but no change of the phosphorylation state of Akt at S473 or Thr308 or the total Akt were observed. However, we observe that the AMP-activated protein kinase (AMPK), an inhibitor of the mTOR protein, was significantly activated by propionate treatment [4], suggesting that AMPK pathway was involved for SCFA to induce autophagy.

AMPK signaling activation induced by propionate was associated with mitochondrial defect-mediated cellular ATP depletion and oxidative stresses. Mitochondria are organelles that primarily produce ATP via oxidative phosphorylation in the inner membrane. Reactive oxygen species (ROS) is an inevitable by-product of mitochondrial metabolism and can cause mitochondrial damage. Such damage subsequently induces the mitochondrial membrane permeability transition (MPT), mitochondria swelling, and cell death. Mitophagy, a selective form of autophagy by which mitochondria are degraded in autolysosomes, plays an essential role in maintaining mitochondrial functional and genetic integrity [22]. Propionate induced ATP reduction and ROS generation in colon cancer cells due to the induction of MPT and loss of the mitochondrial membrane potential. The proportion of mitochondria with lower fluorescence intensity, which represents the depolarized mitochondria, was increased by propionate treatment in a dose- and time-dependent manner in the colon cancer cells. Furthermore, propionate treatment led to co-localization of mitochondria and GFP-LC3 puncta in colon cancer cells. COXIV, a mitochondrial marker, was reduced and localized as defective mitochondria by autolysosomes. An ubiquitin-binding protein-p62, which interacts with LC3 and regulates autophagosome formation, significantly co-localized with mitochondrial COXIV. Consistently, inhibition of autophagic degradation by CQ dramatically increased the accumulation of defective mitochondria [4]. The results suggest the ability of propionate to induce mitophagy, which selectively targets mitochondria with a depolarized membrane potential.

The induction of autophagy by SCFAs may serve as an adaptive strategy for colon cancer cells to retard apoptotic cell death. Application of an autophagy inhibitor was found to enhance the

rate of apoptosis after treatment with SCFAs. Co-treatment of HCT116 cells with propionate/3-MA significantly reduced the percentage of GFP-LC3 formation. Meanwhile, the number of apoptotic cells increased as indicated by the high annexin-V staining. Western blot analysis also indicated the increased cleavages of the pro-apoptotic cascapase-7 and executioner caspase-3. Chloroquine, another inhibitor of autophagy, enhanced apoptosis in HCT116 cells especially at the later stages of treatment. When autophagy was inhibited with molecular shRNA targeted to AMPK$\alpha$ or ATG5, there was a decreased ability of propionate to induce GFP-LC3 puncta formation in HCT116. Consistent with the pharmacologic inhibitory experiment, AMPK$\alpha$ or ATG5 depletion cells displayed a significantly enhanced apoptosis after propionate treatment [4]. The findings suggest that autophagy confers a protective role in propionate-mediated cell death in colon cancer cells.

## 3. Unsaturated fatty acids

An unsaturated fatty acid is a fatty acid in which there is at least one double bond within the hydrocarbon chain. The most common MUFAs in daily nutrition is oleic acid (18:1 n-9), followed by palmitoleic acid (16:1 n-7) and vaccenic acid (18:1 n-7). Oleic acid could increase the ratio of HDL to LDL cholesterol and decrease aggregation of thrombocytes. ω-3 and ω-6 PUFAs are termed as essential fatty acids because they are necessary for health but cannot be synthesized de novo in humans. Autophagy has been reported to be induced by unsaturated fatty acids and involved in the regulation of lipid metabolism in hepatocytes and apoptosis in cancer cells.

### 3.1. Monounsaturated fatty acid

As one of the most abundant fatty acid in the diet and serum, oleic acid (18:9 n-1) is a monosaturated fatty acid with the double bond at its ω-9 position. Autophagy triggered by oleate was observed in mammary epithelial cells, hepatocytes, and osteosarcoma cells [3,23-24]. Furthermore, oleate-triggered autophagy was demonstrated by in vivo experiment. Oleate administered via intraperitoneal injection could cause a rapid autophagic response in the heart, liver, and skeletal muscle of mice characterized by LC3 lipidation, p62 degradation, and phosphorylation of AMPK [23]. Treatment with oleic acid in HepG2 cells significantly increased the number of double-membrane autophagosomes with enveloped cytosolic contents. Meanwhile, oleic acid treatment led to the increase of GFP-LC3 puncta number in a concentration-dependent manner, indicating that oleic acid could induce autophagy in hepatocytes [3]. Oleic acid did not suppress mTOR activity as determined by the level of phosphorylated 4EBP1 and p70S6K at different time points and various concentrations. However, oleic-acid-induced autophagy required ROS formation and the classic PI3 kinase complex. The level of ROS production was increased in oleic-acid-treated cells via activation of the NADPH oxidase enzyme complex [25], and the number of GFP-LC3 puncta formation induced by oleic acid could be markedly suppressed by antioxidant NAC and PI3 kinase inhibitor.

Autophagy triggered by oleic acid contributed to the regulation of lipids metabolism in hepatocytes [3]. Free fatty acids taken up by hepatocytes convert into triglyceride (TG) for storage with cholesterol in lipid droplets (LD). When energy is required, the stored TG is hydrolyzed via activation of lipolytic pathways. Knockdown of autophagic gene ATG5 increased TG levels in hepatocytes cultured with oleate or a second endogenous stimulus for TG formation, methionine- and choline-deficient medium (MCDM). Pharmacological inhibition of autophagy with 3-MA also markedly increased the TG content. Consistent with the increased TG levels, lipid staining revealed increased LD number and size in hepatocytes cultured with oleate or MCDM that were further increased by 3-MA addition or ATG5 knockdown [26]. Autophagy did not change TG synthesis as the comparable increase in TG synthesis occurred in ATG5 knockdown and control cells in response to oleate or MCDM. However, ATG5 knockdown led to much lesser increase in the rate of β-oxidation, inductive of the level of free fatty acids generated by TG hydrolysis as well as significant decrease in TG breakdown in hepatocytes cultured in oleate or MCDM [26]. The regulation of lipid metabolism by autophagy is further supported by the association of autophagic pathway components with LDs. Oleate treatment increased co-localization of LDs with the lysosome-associated membrane protein type 1 (LAMP1). Inhibition of autophagosome formation with 3-MA or autophagosome–lysosome fusion with vinblastine markedly reduced LD/LAMP1 co-localization. Moreover, LD co-localization with autophagosome marker LC3 demonstrates a direct association between LDs and autopaghosomes.

Niso-Santano et al. [23] reported recently that oleate-induced non-canonical autophagy in human osteosarcoma U2OS cells required an intact Golgi apparatus. Oleate treatment promoted the co-localization of LC3 with several proteins of trans-Golgi network including trans-Golgi network protein 2 (TGOLN2) and galactose-1-phosphateuridylytransferase (GALT). Similarly, GFP-LC3 puncta induced by oleate co-localized with lectin mannose-binding 1 (LMAN1), a marker of the endoplasmic reticulum–Golgi intermediate compartment and RAB7A, a protein residing in late endosomes. In addition, transmission electron microscopy assessment confirmed the co-localization of vacuolar structures induced by oleate with the Golgi apparatus to the nuclear periphery. Although the disruption of Golgi apparatus could induce autophagy in human cell lines [27], the structural and functional integrity of Golgi apparatus were not affected by the administration of oleate. Moreover, disruption of Golgi apparatus with brefeldin A reduced the ability of oleate to promote autophagic activity, suggesting that oleate possesses the capacity to stimulate autophagic responses but does not compromise the integrity of the Golgi apparatus.

### 3.2. ω-3 polyunsaturated fatty acids

Omega-3 PUFAs docosahexaenoic acid (DHA, 22:6 n-3) and eicosapentaenoic acid (EPA, 20:5n-3), which are found primarily in cold-water fish and fish oils, reduce the incidence and progress of a series of human diseases including cancer. The ability of DHA and EPA to induce autophagy has been reported in myocardioblasts and diverse human cancer cells [28-32]. DHA treatment dramatically increased the formation of visualized autophagic vacuoles and LC3 puncta. The number of lysotracker-positive vesicles as well as the co-localization of LC3 with

lysotracker was also markedly increased. Chloroquine promoted the induction of LC3-I by DHA. These results collectively suggest that DHA triggers autophagy via activation of the autophagic flux [30]. Dietary DHA and EPA could be converted to their ethanolamide derivatives, docosahexenoyl ethanolamine (DHEA) and eicosapentaenoyl ethanolamine (EPEA). The level of autophagy gene Beclin-1 in breast cancer cell MCF-7 could be significantly increased after treatment with DHEA or EPEA. Furthermore, ethanolamides reduced the association between Beclin-1 and Bcl-2, indicating that DHEA and EPEA could induce autophagy in human breast cancer cells [33].

The molecular mechanism underlying DHA-triggered autophagy is related to the p53/AMPK/mTOR signal pathway. The tumor suppressor p53 plays a dual role in the control of autophagy. On one hand, nuclear p53 can induce autophagy through transcriptional effects. On the other hand, cytoplasmic p53 may acts as a master repressor of autophagy [34]. Tissues from adult mice with an expression of a GFP-LC3 transgene on a p53–/– background showed a higher level of GFP-LC3 puncta than p53+/+ and heterozygous littermate. Consistently, inhibition of p53 with siRNA or pharmacological inhibitor in human cancer cell induced the accumulation of GFP-LC3 puncta. In human cervical and breast tumor cell lines, DHA dose-dependently downregulated the level of p53 and triggered autophagy through a signaling pathway similar to p53 inhibition mediated autophagy. When p53 degradation was inhibited by MG132, the accumulation of GFP-LC3 puncta induced by DHA was significantly reduced, indicating that loss of p53 is responsible for DHA-induced autophagy in cancer cells.

The AMPK/mTOR signaling pathway is responsible for p53 inhibition mediated autophagy. In p53–/– cells, AMPK and its substrates tuberous sclerosis complex 2 (TSC2) and acetyl CoA carboxylase (ACC$\alpha$) were hyperphosphorylated whereas p70S6K was hypophosphorylated. siRNA-mediated depletion of AMPK$\alpha$1 and AMPK$\alpha$2 or inhibition of mTOR with rapamycin eliminated the difference in autophagy between p53-inhibited and control cells [34]. DHA treatment increased the level of phosphor–AMPK and phosphor–ACC$\alpha$ expression and decreased the activity of mTOR pathway, suggesting that DHA induces autophagy through p53-mediated AMPK/mTOR signaling.

Autophagy induced by EPEA and DHEA in breast cancer cells has been attributed to the activation of proliferator-activated receptor gamma (PPAR$\gamma$). DHA and EPA as well as their ethanolamide derivatives act as the activator of PPAR$\gamma$, which has been reported to induce autophagy in breast cancer cells [35]. PPAR$\gamma$ modulates the transcription of phosphatase and tensin homolog on chromosome ten (PTEN), a unique phosphatase with the ability to decrease the levels of phosphorylated AKT and consequently AKT-mediated pathway [36]. Via trans-activation of PPAR$\gamma$ in MCF7 cells, DHEA and EPEA enhanced PTEN protein levels and subsequently decreased the activity of AKT/mTOR signaling pathway which is responsible for the induction of autophagy [33].

Omega-3 PUFAs can induce apoptosis by activating both intrinsic and extrinsic apoptotic pathways. DHA-induced autophagy contributes to the cytotoxic activity by enhancing apoptosis in cancer cells. While it is generally accepted that autophagy function as a mechanism for cells to survive from stresses, there is also a substantial body of literature suggesting

that autophagy can promote cell death under certain circumstances [37,38]. Autophagy might degrade cellular components so that the cell eventually activates the apoptosis machinery. DHA-induced apoptosis could be partially blocked when autophagy was inhibited by 3-MA. Consistently, ATG5 and ATG7 knockdown by siRNA reduced both autophagy and apoptosis response to DHA treatment in human cancer cells, indicating that autophagy enhances DHA-induced apoptosis and inhibition of autophagy prevents DHA-apoptotic cell death [30].

### 3.3. ω-6 polyunsaturated fatty acids

Omega-6 PUFAs are characterized by the presence of at least two carbon–carbon double bonds with the first bond at the sixth carbon from the methyl terminus. The ability of omega-6 PUFA to induce autophagy was observed in *C. elegans* [39]. As in mammalian cells, autophagy occurs at basal levels during normal growth conditions in *C. elegans*, but is rapidly upregulated in response to certain environmental stresses. Using *C. elegans* that express the GFP::LGG-1 reporter as an integrated transgene, autophagy was found to be activated by starvation in the pharyngeal muscle. Enrichment of ω-3 PUFA EPA and ω-6 PUFAs arachidonic acid (AA, 20:4 n-6) and di-homo-γ-linoleic acid (DGLA, 20:3 n-6) was observed in fasted *C. elegans*. Supplementation with AA and DGLA, but not with EPA, was sufficient to activate autophagy in *C. elegans*, indicating the role of ω-6 PUFAs in starvation-triggered autophagy. The *C. elegans* fatty acid desaturases fat-6 and fat-7 are required for long-chain unsaturated fatty acids synthesis, and fat-1 is required for the conversion of ω-6 to ω-3 PUFAs. RNAi targeted to fat-6, fat-7, or fat-1 does not affect the intensity or distribution of LGG1 puncta in well-fed animals, suggesting that the effects of ω-6 PUFAs on autophagy is achieved only when their levels relative to fatty acids are above a certain threshold, which cannot be recapitulated by inactivating the enzymes responsible for the synthesis of ω-6 PUFAs or their conversion to ω-3 PUFAs [39].

Autophagy can contribute lifespan extension of *C. elegans*. Mutations in the insulin-like growth factor (IGF-1) receptor DAF-2 display an increase in adult *C. elegans* longevity. However, autophagy inactivation by RNAi of BEC-1 (the ortholog of Beclin-1), ATG7, and LGG3 (the ortholog of ATG12) decreased lifespan of DAF-2 mutants, indicating that autophagy genes are required for lifespan extension [40]. Dietary restriction plays an evolutionarily conserved role in lifespan extension in yeasts, flies, mammals, and *C. elegans*. The correlation between increased autophagy and lifespan in feeding-defective *C. elegans*, *eat-2*, *eat-3*, and *pha-3*, provides a clue that autophagy might be involved in dietary restriction-mediated lifespan extension [41,42]. Chronic long-term dietary supplementation with AA and DGLA extended *C. elegans* lifespan under the condition of food abundance. Inactivation of the essential autophagy genes BEC-1, LGG-1 (the ortholog of ATG8/MAPLC3), and ATG16.2 (the ortholog of ATG16p/ATG16l1) suppressed the life extension induced by ω-6 PUFAs. These results collectively revealed that ω-6 PUFAs increased *C. elegans* lifespan through the activation of autophagy in well-fed condition. While the mechanisms by which autophagy mediates lifespan extension are not yet understood, the possibility is that autophagy removes damaged mitochondria, decreased levels of intracellular reactive oxygen species, and subsequently protects against oxidative damage. Many of the long-lived mutants in *C. elegans* were resistant to oxidative stress and many mutations that decrease mitochondrial electron transport are

long-lived whereas, conversely, mutations that increase oxidative damage shorten lifespan in *C. elegans*. Thus, longevity in *C. elegans* may be mediated either by mutations that directly affect cellular generation or breakdown of reactive oxygen species or indirectly decrease reactive oxygen specified via upregulation of autophagic turnover of damaged organelles that generate these harmful species.

ω-6 PUFAs are supposed to induce autophagy in mammalian cells. Dietary supplementation of ω-6 PUFAs improve several human conditions that overlap with the pathologies associated with autophagy malfunctions such as chronic inflammation, neuro-degenerative disease, and cancer. Supplementation with AA and DGLA but not EPA leads to the increase of autophagic marker LC3-II and the formation of LC3 puncta in HeLa cells accompanied with the decreased level of p62 [39]. Furthermore, the number of LC3 puncta in HeLa cells treatment with AA or DGLA could be augmented by the inhibition of lysosomal enzymes and impaired by the inactivation of autophagy gene ATG161L indicated the ability of ω-6 PUFAs to trigger autophagy in HeLa cells via activation autophagic flux.

### 3.4. Trans fatty acids

Trans fatty acids (TFA) have one or more double bonds in *trans* configuration instead of the usual *cis* configuration. The primary dietary trans TFAs are vaccenic acid (18:1 n-7t) and elaidic acid (18:1 n-9t). Vaccenic acid is a naturally occurring TFA found in the fat of ruminants and diary products, whereas elaidic acid is the major TFA found in hydrogenated vegetable oils [43]. TFAs increase LDL and decrease the beneficial HDL levels resulting in a less desirable LDL/HDL ratio, indicating the deleterious effects of TFAs consumption on human health. Extensive evidence has proved the direct connection of TFAs with coronary heart disease, cancer, Alzheimer's disease, diabetes, and other diseases. TFAs-induced autophagy in primary rat cardiac myofibroblasts has been recently reported [44]. Both vaccenic acid and elaidic acid could induce autophagosome formation, LC3 lipidation, LC3-II formation, increased beclin-1 concentration and ATG5–ATG12 complex formation. Considering that inhibition of lysosome–autophagosome fusion with bafilomycin A1 further increased TFAs-mediated LC3-II formation, the authors believe that TFAs-triggered autophagy is related to de novo autophagosome formation and subsequent turnover [44].

TFAs treatment led to marked apoptotic cell death of primary cardiac myofibroblasts, which is dependent upon the activation of autophagy [44]. Both vaccenic acid and elaidic acid induced cell death in a concentration-dependent manner. Meanwhile, caspase-3 and 9, but not caspase-8 or Bid, were activated by TFAs treatment, indicating that the intrinsic apoptosis was induced by TFAs. The role of autophagy in TFAs-induced apoptosis was confirmed in ATG3 and ATG5 knockout mouse embryonic fibroblasts (MEFs). Absence of ATG3 and ATG5 significantly reduced the cytotoxic effects of TFAs. Moreover, ATG3 or ATG5 knockout decreased the activation of caspase 3 and caspase 7 induced by TFAs exposure, demonstrating that autophagy is necessary for TFAs-induced apoptotic cell death [44]. As TFAs treatment led to the generation of ROS and decreased mitochondrial membrane potential [45], it has been hypothesized that the mechanism underlying the interplay between autophagy and apoptosis upon TFAs treatment may be related to the disruption of the mitochondrial metabolism and

membrane potential which serves as a powerful trigger for the induction of apoptosis and autophagy.

### 3.5. Conjugated fatty acids

Conjugated fatty acids (CFAs) are a mixture of positional and geometric isomers of polyunsaturated fatty acids with conjugated double bonds. The most abundant fatty acids with a conjugated system of double bonds are isomers of linoleic acid (conjugated linoleic acid, CLA), which are found mostly in the meat and dairy products derived from ruminants. Several CLA isomers exist due to positional and geometrical isomerism of the conjugated double bonds and the major naturally existing one of which is referred to as 9Z11E-18:2 [1]. Most recently, CLA has been reported to induce nonalcoholic steatohepatitis in an animal model. The liver of mouse feeding with CLA-containing diet displayed more LC3-positive cells compared with those fed with control diet, indicating the ability of CLA to increase autophagy in liver cells [46].

In addition to CLA, many other CFAs occur naturally in plant seeds and marine algae. Alpha-eleostearic acid (ESA, 9Z11E13E-18:3) is a linolenic acid isomer with a conjugated triene system. ESA makes up approximately 80% of the fatty acids in tung oil and 60% of bitter gourd seed oil. Autophagy induction by ESA was observed in Hela cells as evidenced by the autophagic vacuoles as well as the conversion of LC3-I to LC3-II [47]. ESA decreases phosphorylation of P70KS and AKT effectively in both time- and dose-dependent manner. On the contrary, ERK1/2 phosphorylation could be increased by ESA treatment, indicating that autophagy induced by ESA is involved in the regulation of ERK1/2 and AKT/mTOR signal pathway [47]. Although ESA possesses strong suppressive effects on tumor growth, the induction of autophagy by ESA functions as a protective mechanism against cell death in HeLa cells. When autophagy was inhibited with 3-MA, the viability of HeLa cell was markedly reduced in response to ESA treatment [47].

## 4. Conclusion

Fatty acids are important molecules with multiple physiological properties including serving as major metabolic fuel, essential components of all membranes, and gene regulators. As an evolutionarily conserved catabolic process, autophagy is generally thought as a survival mechanism in dealing with stress by clearing damaged proteins, organelles, or pathogens or by providing the cells with energy and anabolic building blocks during starvation. Autophagy could be triggered by fatty acids and contributes to the pathologic and physiological processes mediated by fatty acid such as the metabolism of lipid, lipotoxicity, life extension, and antitumor activity (Figure 1). While the regulation of ROS generation, ceramide synthesis, and multiple signal pathways like AKT/mTOR, JNK, and PKC are involved in fatty-acids-induced autophagy, the molecular mechanisms involved and the implications in different physiological or pathological processes need to be further elucidated.

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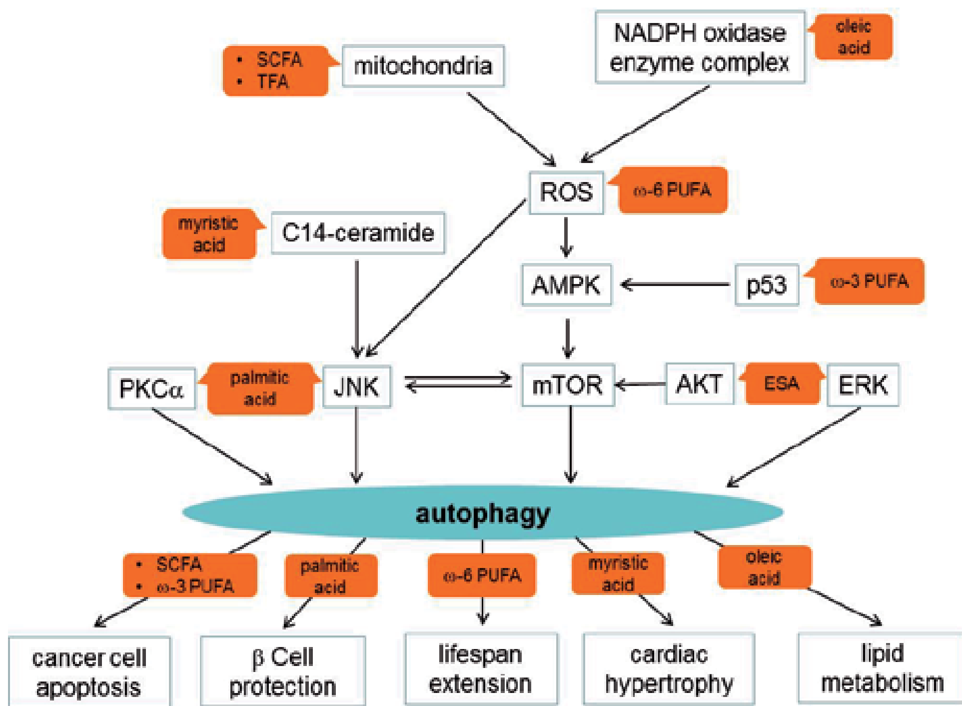


**Figure 1.** The regulation of autophagy by fatty acids and its biological significances.

Multiple signaling pathways are involved in fatty-acids-mediated autophagy. JNK is responsible for autophagy triggered by long-chain saturated fatty acids, palmitic acid and myristic acid. ROS plays an essential role in autophagy regulation through AMPK/mTOR pathway. SCFA, TFA, oleic acid, and ω-6 PUFA-mediated autopahgy is attributed to the increase of ROS via disruption of mitochondrial metabolism, or activation of NADPH oxidase enzyme complex. ω-3 PUFAs induce autophagy through p53-dependent AMPK/mTOR signaling. Other signaling pathways such as AKT/mTOR and PKCα are also involved in autophagy regulation by fatty acids. Autophagy triggered by fatty acids contributes to diverse pathological and physiological processes including apoptosis of cancer cells, β cell protection and insulin resistance, life extension, lipid metabolism, and cardiac hypertrophy.

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Chapter 9

# Autophagy as a Therapeutic Target in Gastrointestinal Cancer

Michiko Shintani

Additional information is available at the end of the chapter

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**Abstract**

Autophagy is a bulk protein and organelle degradation system and is an important homeostatic cellular recycling mechanism. The following kinds are the three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. In general, the term "autophagy" indicates macroautophagy. Autophagy is mediated by double-membrane-bound structures called autophagosomes. During the autophagic process, cytoplasmic components are sequestered and engulfed by autophagosomes. Autophagosomes then fuse with lysosomes to form autolysosomes where the sequestered components are digested by lysosomal hydrolases. Microtubule-associated protein 1 light chain 3 (LC3) is an autophagosomal ortholog of the yeast protein ATG8. Autophagy stimulates the upregulation of LC3 expression, and a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-II which is recruited to autophagosomal membranes. Subsequently, LC3-II is degraded by lysosomal hydrolases after the fusion of autophagosomes with lysosomes. Therefore, LC3 is a specific marker of autophagosome formation. Additionally, beclin 1, the mammalian ortholog of the yeast protein ATG6, has been known to play a crucial role in autophagy. Beclin 1 acts in conjunction with the phosphoinositide-3 kinase pathway to enhance the formation of the autophagic vacuole.

Recently, autophagy has been reported to play roles in both cell death and survival. Autophagy is a multifaceted process, and alterations in autophagic signaling pathways are frequently observed in cancer. Cancer is a disease caused by mutation, selection, and genome instability in tumor tissues, and the role of autophagy in cancer is unclear.

One anticancer treatment strategy is to trigger tumor-selective cell death. Apoptosis is regarded as the central mediator of programmed cell death in response to radiation and chemotherapy. Our previous report suggested that different cell-death pathways are activated in gastric and colorectal carcinomas and the extrinsic and

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intrinsic apoptotic pathways could be mutually regulated in gastric adenocarcinomas. In contrast, in colorectal carcinomas, autophagy may function as a cellular guardian to prevent caspase-9-dependent apoptosis (intrinsic apoptotic pathway). LC3 positivity was less frequent in gastric adenocarcinomas than in colorectal adenocarcinomas. Therefore, we suggested that LC3 expression in colorectal carcinomas is likely to aid cancer therapy, owing to its involvement in apoptosis and/or autophagy.

In this chapter, we discuss the following: (1) the detection of autophagy using immunohistochemistry, (2) autophagy and tumor suppression and/or progression, and (3) autophagy as a therapeutic target in gastrointestinal carcinomas.

**Keywords:** Gastric carcinoma, colorectal carcinoma, immunohistochemistry, cancer therapy

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## 1. Introduction

Autophagy is a bulk protein and organelle degradation system and is an important homeostatic cellular recycling mechanism. The following are the three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. In general, macroautophagy is believed to be the major type of autophagy. Autophagy is mediated by double-membrane-bound structures called autophagosomes [1–3]. During the autophagic process, cytoplasmic components are sequestered and engulfed by autophagosomes. Autophagosomes then fuse with lysosomes to form autolysosomes where the sequestered components are digested by lysosomal hydrolases. Microtubule-associated protein 1 light chain 3 (LC3) is an autophagosomal ortholog of the yeast protein ATG8. LC3 exists in two forms, LC3-I and LC3-II. LC3-I is localized in the cytoplasm. Autophagy stimulates the upregulation of LC3 expression, and LC3-I conjugates with phosphatidylethanolamine to form LC3-II. LC3-II binds to autophagosomes and it is degraded by lysosomal hydrolases after the fusion of autophagosomes with lysosomes [4–6]. Therefore, LC3 is a specific marker of autophagosome formation. The autophagic pathway includes several phases: initiation, vesicle elongation, maturation, fusion, and degradation (Figure 1). Recent studies have suggested that additional membranes are derived from the Golgi complex, mitochondria, and plasma membrane; however, this phenomenon has not been confirmed [1–2, 7–10].

Current studies are examining the molecular regulation and function of autophagy. Additionally, autophagy is believed to play a role in various diseases such as cancer, infectious diseases, cardiovascular diseases, metabolic diseases, pulmonary diseases, and neurodegenerative disorders [11–20]. Recently, in clinical trials, several autophagic inhibitors, including hydroxychloroquine and chloroquine, have been examined as targets in diseases. In cancer, these autophagic components are being studied to enhance chemotherapeutic efficacy. Thus, autophagy is now an important and widely studied topic in human health and disease [11–15].

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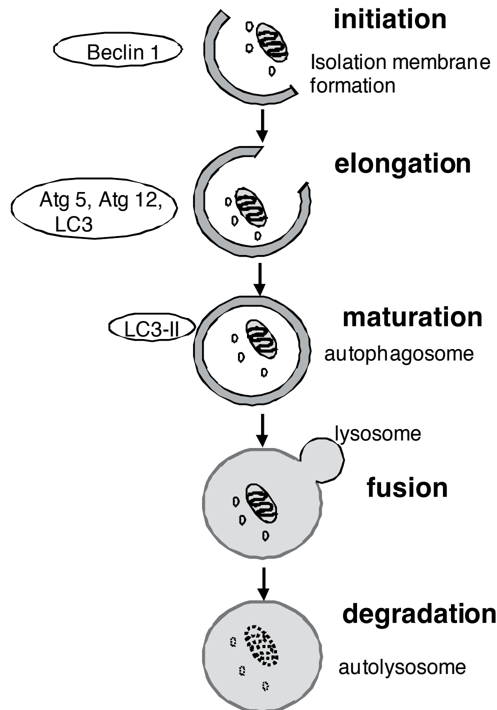


**Figure 1.** Cellular mechanism of autophagy. The autophagic pathway includes several phases: initiation, vesicle elongation, maturation, fusion, and degradation.

## 2. Detection of autophagy using immunohistochemistry

The role of autophagy in cancer development and progression has been studied by investigating autophagy-related proteins, LC3, beclin 1, and p62 using immunohistochemistry [21–23].

### 2.1. LC3

LC3 is an autophagosomal ortholog of the yeast protein ATG8 and is a specific marker of autophagosome formation. LC3-I is localized in the cytoplasm whereas LC3-II binds to autophagosomes. LC3 is presently used as an autophagy marker [21–26].

### 2.2. Beclin 1

Beclin 1 is the mammalian homolog of the yeast protein ATG6 and it has a central role in autophagy. The expression of beclin 1 has been reported in tumors such as breast, ovarian, prostate, lung, brain, stomach, and colorectal tumors. Beclin 1 may play a role in the tumorigenesis and/or progression of human cancers. However, beclin 1 has several physiological functions other than autophagy [21–22, 27–31].

### 2.3. p62

p62/SQSTM1 (p62) is an autophagy substrate protein which accumulates in autophagy-deficient cells after metabolic stress. p62 accumulation leads to mitochondrial damage, oxidative stress, and DNA damage. Additionally, p62 accumulation has been strongly suggested to result in cancer development [23, 32].

## 3. Autophagy and tumor suppression/progression

Recently, several studies have suggested that dysregulation of autophagy plays a critical role in tumorigenesis. Liang et al. (1999) reported that beclin 1 can inhibit tumorigenesis and that its levels decrease in human breast carcinoma [27]. Qu et al. (2003) presented the genetic evidence for the role of autophagy genes in tumor suppression. They suggested that beclin 1 is a haploinsufficient tumor-suppressor gene, and that mutation of beclin 1 or other autophagy genes may contribute to the pathogenesis of human cancers [28]. Autophagy may prevent normal cells from developing into tumor cells; however, it may also protect cancer cells by providing nutritional support. Yang et al. (2011) investigated the effects of autophagy in stressed and unstressed colon cancer cells. They found that, in unstressed cells, the inhibition of autophagy was associated with a significant growth advantage but, in biologically stressed cells, the inhibition of autophagy markedly reduced cell viability compared to that observed in controls. Therefore, they suggested that autophagy has a dual role in colon cancer cells; it is pro-survival under biological stress and pro-death under normal conditions [33].

Li et al. (2009) reported the dual role of autophagy in colon cancer. They found that the inhibition of autophagy enhances 5-fluorouracil-induced colon cancer cell apoptosis and improves the chemotherapeutic effect of 5-fluorouracil. This result indicates that autophagy plays a role in protecting some cells from chemotherapy-induced death [34].

Autophagy may play an important role in maintaining normal cellular homeostasis and may prevent normal cells from developing into cancer cells. However, autophagy is a cellular recycling mechanism and is active during metabolic stress [35–36]. Additionally, it may prevent cell death in tumor cells (apoptosis or autophagic cell death). Thus, autophagy has a role in both the suppression of cancer initiation and the promotion of cancer growth [27–28, 33–34].

## 4. Autophagy as a therapeutic target in gastrointestinal carcinomas

Autophagy has a role in both tumor promotion and tumor suppression. In cancer therapy, using the effect of autophagy, cell survival is inhibited or cell death is promoted by inhibition or induction [37–41].

Recently, autophagy has been used for cancer therapy in aggressive cancers. Particularly, a clinical trial of cancer therapy involving the combination of an autophagy regulator with

conventional anticancer agents or radiation therapy has been performed. In colorectal cancer, a combination of an autophagy inhibitor (hydroxychloroquine), oxaliplatin, leucovorin, 5-fluorouracil, and bevacizumab has been used. Several studies have reported the influence of apoptosis and autophagy on each other in cancer cells after chemotherapy [42–45].

Therapeutic agents in gastrointestinal cancer are summarized in Tables 1 and 2 [46–57].

## 5. Conclusion

Autophagy, an intracellular process involved in removing and recycling cellular components, may play a role in both protecting and promoting cancer cell death under different stress situations. The role of autophagy in tumorigenesis is controversial, because it can either protect or promote cell death. Recently, autophagy has been used in cancer therapy for aggressive cancers. Particularly, a clinical trial of cancer therapy involving the combination of an autophagy regulator with conventional anticancer agents or radiation therapy has been performed [5, 12, 37–41].

Our previous study suggested that different cell-death pathways are activated in gastric and colorectal carcinomas and the extrinsic and intrinsic apoptotic pathways could be mutually regulated in gastric adenocarcinomas. In contrast, in colorectal carcinomas, autophagy may function as a cellular guardian to prevent caspase-9-dependent apoptosis (intrinsic apoptotic pathway). LC3 positivity was less frequent in gastric adenocarcinomas than in colorectal adenocarcinomas [25]. Therefore, we suggested that LC3 expression in colorectal carcinomas is likely to aid cancer therapy. The detection of apoptosis and autophagy activity may help predict the treatment effect in colorectal cancer.

Presently, the anticancer agents that induce apoptosis are mainly being used. The development of a drug that induces autophagic cell death is expected in the near future. Therefore, the identification of a marker for determining the autophagic effect of drugs is important. Additionally, the development of a regulator specific for autophagy is needed.

| Alisertib (ALS) | Yuan etal. [46] |
|---|---|
| ALS exerts potent inhibitory effects on cell proliferation, and inducing effects on cell-cycle arrest, mitochondria-dependent apoptosis, and autophagy with the involvement of PI3K/Akt/mTOR, p38 MAPK, and AURKA-mediated signaling pathways in AGS and NCI-N78 cells. | |
| Compound 1 | Chun et al. [47] |
| Compound 1 effectively inhibits the growth of AGS cells by inducing apoptosis, as well as autophagy. Apoptosis after compound 1 treatment is associated with activation of caspases, release of cytochrome c, and an increased ratio of Bax/Bcl-2. Autophagy with compound 1 treatment is indicated by LC3-II protein expression. | |
| *Klotho* gene (klotho protein) | Xie et al. [48] |

<table>
<tr><td colspan="2">Klotho is a tumor suppressor in gastric cancer. Restoration of klotho gene expression significantly inhibits cell proliferation, induces cell apoptosis, and increases LC3-I/LC3-II expression in gastric cancer cells. Klotho regulates IGF-1R phosphorylation and the subsequent activation of IRS-1/PI3K/Akt/mTOR signaling, tumor cell proliferation, apoptosis, and autophagy.</td></tr>
<tr><td>Evodiamine</td><td>Rasul et al. [49]</td></tr>
<tr><td colspan="2">Evodiamine significantly inhibits the proliferation of SGC-7901 cells and induces G2/M phase cell cycle arrest. Furthermore, both autophagy and apoptosis are activated during the evodiamine-induced death of SGC-7901 cells. Evodiamine is an effective natural compound for the treatment of gastric cancer, and it may be used in in vivo studies of monotherapies or combined antitumor therapies.</td></tr>
</table>

**Table 1.** Therapeutic agents in gastric cancer

<table>
<tr><td>Melphalan, bortezomib, and rapamycin</td><td>Song et al. [50]</td></tr>
<tr><td colspan="2">Melphalan triggers apoptosis, bortezomib induces apoptosis and autophagy, and rapamycin induces autophagy. The combination treatment induces synergistic apoptosis, which is mediated through an increase in caspase activation in human colon cancer cell lines. Mitochondrial dysfunction induced by this combination treatment has been linked with altered cellular metabolism, which induces adenosine monophosphate-activated protein kinase (AMPK) activation. AMPK-induced apoptosis, through an interplay between autophagy and apoptosis, is triggered by this combination treatment.</td></tr>
<tr><td>DENSpm</td><td>Çoker-Gürkan et al. [51]</td></tr>
<tr><td colspan="2">The effect of DENSpm, a polyamine analog, on cell death differs according to the p53 protein expression profile. In addition, DENSpm-induced autophagy may be critical in drug resistance in colon cancer cells.</td></tr>
<tr><td>Apigenin (4',5,7-trihydroxyflavone)</td><td>Lee et al. [52]</td></tr>
<tr><td colspan="2">Apigenin is a natural flavonoid with apoptosis- and autophagy-inducing effects in HCT116 colon cancer cells. Autophagy plays a cytoprotective role in apigenin-induced apoptosis, and the combination of apigenin and an autophagy inhibitor may be a promising strategy for colon cancer.</td></tr>
<tr><td>Compound K (20-O-(β-D-glucopyranosyl)-20(S)-protopanaxadiol)</td><td>Kim et al. [53]</td></tr>
<tr><td colspan="2">Compound K stimulates autophagy as well as apoptosis by disrupting the interaction between Atg6 and Bcl-2. The induction of autophagy and apoptosis by compound K is mediated through reactive oxygen species generation and c-Jun NH2-terminal kinase activation in human colon cancer cells.</td></tr>
<tr><td>LBH589</td><td>Gandesiri et al. [54]</td></tr>
<tr><td colspan="2">The histone deacetylase inhibitor (HDACi) LBH589 has been verified as an effective anticancer agent. Death-associated protein kinase (DAPK) induces autophagy in response to HDACi treatment. In autophagy-deficient cells, DAPK plays an essential role in committing cells to HDACi-induced apoptosis.</td></tr>
<tr><td>MS-275</td><td>Zhan et al. [55]</td></tr>
<tr><td colspan="2">MS-275 is a synthetic benzamide derivative of an HDACi. p38 MAP kinase plays a vital role in the switch from autophagy to apoptosis in MS-275-induced human colon cancer cells. High expression of p38 induces cell autophagy,</td></tr>
</table>

| | |
|---|---|
| but low expression results in apoptosis. MS-275 may be a promising clinical chemotherapeutic agent with multiple effects. | |
| 11'-deoxyverticillin A (C42) | Zhang et al. [56] |
| C42 is an epipolythiodioxopiperazine. C42 enhances the cellular autophagic process, which requires both PARP and receptor-interacting protein 1 participation, and this precedes and possibly augments caspase-dependent apoptotic cell death. | |
| Sulforaphane (SUL) | Nishikawa et al. [57] |
| SUL, a type of isothiocyanate and a pro-apoptotic agent, triggers the induction of autophagy by endothelial cells, similar to cancer cells, and the inhibition of autophagy potentiates the pro-apoptotic effect. This suggests the possible use of autophagy inhibitors in combination with anti-angiogenic agents. | |

**Table 2.** Therapeutic agents in colorectal cancer

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Section 2

# Apoptosis

Chapter 10

# Apoptotic Molecular Advances in Breast Cancer Management

Pontsho Moela and Lesetja R. Motadi

Additional information is available at the end of the chapter

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**Abstract**

Breast cancer is the most common cancer type amongst women, accounting for most female cancer deaths second to cervical cancer worldwide. It is, therefore, highly crucial to understand the molecular biology and explore other pathways involved in carcinogenesis in order to select appropriate treatment not only for breast cancer but for other cancers as well. Cancer progression is favoured by DNA damage and in most cases a consequent disruption of the apoptotic pathway, thus leading to uncontrolled cell proliferation. Therefore, current therapeutic strategies aim at targeting the apoptotic pathways in order to combat cancer. In this manuscript, we discuss the ways in which evasion of apoptosis during carcinogenesis occurs and the types of current therapeutic strategies as well as promising future approaches against breast cancer.

**Keywords:** Breast cancer, apoptosis, small molecules, p53, RBBP6

## 1. Introduction

The human body is composed of trillions of cells that behave and function to provide structure of the body, convert nutrients into energy and carry out specialised functions [1, 3]. Growing, dividing, differentiating and dying are the cells' behavioural mechanisms to maintain tissue homeostasis [3]. However, molecular disturbances that disrupt this balance may potentially lead to disease. Such molecular disturbances include mutations, among others, during which any change to the DNA sequence might result in abnormality in the cell or tissue [4]. With a population of more than a trillion cells, the human body is prone to mutations that may give one cell a selective advantage of growing and dividing more vigorously to become a growing mutant clone [4, 5]. Such mutations, in which a mutant clone of cells grows and divides out of control at an expense of neighbouring wild-type cell populations, serve as a prerequisite for the development of cancer [3].

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Cancer is defined as uncontrolled cell proliferation that leads to the formation of abnormal cells and invasion of other adjacent tissues [1, 3-5]. The migration of cells from the origin of tumour to another part of the body is referred to as metastasis. Tumours can either be malignant or benign. While malignant tumours have the ability to invade surrounding tissue, benign tumours cannot invade other tissues and are therefore not as life-threatening [3]. Efficient treatment against malignant tumours is therefore necessary in cancer management. In this chapter, we discuss current anticancer strategies that are targeted on the apoptosis pathway in breast cancer management.

In order to understand breast cancer, it is necessary to understand the normal anatomy of the female breast [3, 20]. The female breast is made up of milk-producing glands called lobules which are connected to ducts that transport milk from the glands to the nipples. The ducts and lobules are surrounded by connective tissue, fatty tissue, blood vessels and lymphatic vessels. In most cases, breast cancer starts in cells surrounding the ducts or the lobules [23]. Metastatic breast cancer is as a result of migration of cancerous cells from ducts and/or lobules via lymphatic vessels to the lymph nodes of the lymphatic system [3, 20, 23].

Breast cancer is the most common cancer type amongst women accounting for many cancer deaths, second to cervical cancer. Risk factors of breast cancer are divided into non-modifiable and modifiable factors [39]. Advanced age, female gender, menarche before the age of 12, menopause after the age of 45, genetic mutations and family history are the major non-modifiable risk factors associated with breast cancer [6, 11, 26, 46, 56, 57]. Breast cancer risk factors that can be controlled include hormone replacement therapy, oral contraceptives, pregnancy, breast feeding and high breast density [31]. Behavioural and life-style risk factors associated with the development of breast cancer include poor diet, i.e. high fat, low vegetable/ fruit, low fibre and high in simple carbohydrates; overweight and obesity; and decreasing physical activity [29, 39].

Nearly 80% of human breast cancers are hormone-positive (estrogen and progesterone), followed by human epidermal growth factor receptor 2 (HER2)-positive, then vascular endothelial growth factor (VEGF)-positive breast tumours [8, 9]. Targeting estrogen receptor (ER) pathway, VEGF and HER2 are the long-established breast cancer therapeutic approaches responsible for the improvements of breast cancer prevention and treatment. However, resistance to these endocrine and cell-growth-inhibiting treatments is the main drawback that reduces the benefits of these novel treatment approaches [8, 9, 20, 23]. It is therefore highly crucial to understand the molecular biology and explore other pathways involved in carcinogenesis in order to select appropriate treatment not only for breast cancer but for other cancers as well. In this chapter we discuss different ways of targeting apoptosis in breast cancer management.

## 2. Targeting apoptosis in breast cancer treatment

During the process of breast cancer progression, normal cells transform into malignant types as a result of genetic alterations [12]. This leads to dysregulation of cellular processes such as

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angiogenesis, cell cycle and apoptosis [17]. Therefore, current therapeutic strategies aim at targeting these pathways, more especially apoptosis, in order to combat cancer [18]. Apoptosis is a form of programmed cell death in which cells are programmed to die if found to be cellular damaged [21, 25]. Apoptosis is made up of two major pathways called the death receptor pathway and the mitochondrial pathway, which are both propagated by a caspase cascade that ultimately leads to apoptosis induction [27, 34]. Evasion of apoptosis during carcinogenesis occurs by three distinct mechanisms: disrupted signalling of death receptors, loss of caspase activity as well as impaired balance between anti-apoptotic and pro-apoptotic proteins [14, 42, 50, 59]. Targeting the caspase cascade, Bcl-2 family proteins as well as other factors associated with apoptosis signalling have thus become the major strategy in anticancer therapeutics (table 1).

| Reagent | Target | Technology | Function | Status |
|---|---|---|---|---|
| **Apoptin** | Caspases in the extrinsic pathway | Vector-based (adenoviral and virus vectors) | Caspase 3 and 8 activation | Preclinical |
| **Flavipirodol, gossypol, depsipeptide, ABT-737, ABT-264, fenretinide, HA 14-1, GX15-070** | Anti-Bcl-2 family proteins | Small molecule | Inhibit BCl-2 family proteins by reducing their expression | Phase I/II |
| **ABT 737** | Anti-apoptotic proteins | Small molecule | Inhibit expression of anti-apoptotic proteins such as Bcl-xL, Bcl-2 and Bcl-W | Phase I |
| **Oblimersen Sodium** | Anti-Bcl-2 targeted drug | Antisense | Bcl-2 antisense increases survival rates in chronic myeloid leukaemia patients when combined with chemotherapy | Phase II |
| **ONYX-015 drug** | p53-based gene therapy | Adenoviral | Genetically engineered adenovirus that has been modified to infect and lyse p53-deficient cells | Phase III |
| **$CD8^+$ cytotoxic T-lymphocytes (CTLs)** | Tumour associated antigens (mutant p53) | Vaccine | Recognize TAA-derived peptides that are processed and presented on the tumours cell surface in association with MHC class I molecules, leading to killing of tumour cells | Phase I |

| Reagent | Target | Technology | Function | Status |
|---|---|---|---|---|
| Phikan083, CP-31398 | p53-targeted | Small molecule | Restores p53 function by intercalating with p53-bound DNA and destabilising the p53-DNA interaction | Phase I/II |
| **Tenovins, Nutlins, MI-219** | p53-MDM2 interaction | Small Molecule | Interrupt the p53-MDM2 interaction to prevent inactivation of p53 by MDM2 | Phase I/II |
| **siMDM2, siE6/7, siBBP6** | p53-MDM2, p53-E6, p53-RBBP6 interaction | Liposomal encapsulated synthetic siRNA | Interrupt p53 interaction with its negative regulators | Research |

**Table 1.** Apoptosis-based anticancer drugs in development

## 2.1. Caspase-targeted therapy

Pathogenic as they are, disruptions in the apoptotic pathway provide compelling possible strategies for the treatment of breast cancer and other related types of cancers [59]. Therapeutic agents designed to re-establish the normal functioning of the apoptotic signalling pathways have the potential to get rid of over 50% of human cancers including breast cancer [34]. Novel drug discoveries in recent years have led to promising advances in the treatment of breast cancer as well as other cancers. For example, the caspase-targeting therapies that use small molecules to act as caspase activators have been identified [24, 32]. These small molecule caspase activators are pro-apoptotic due to their characteristic arginine-glycine-aspartate motif that enables them to directly convert non-active procaspase-3 into active caspase-3 thus leading to apoptosis induction.

Apoptotin is a caspase-based drug therapy that has the ability to induce caspase activity thus increasing apoptosis induction [32]. MCF-7 breast cancer cells completely lack the expression of caspase-3 due to frame-shift mutation in exon 3 of the caspase-3 gene [13]. As a result, caspase-based gene therapy that relies on caspase-3 gene delivery techniques in order to up-regulate caspase-3 expression in caspase 3-deficient breast cancers has been invented. In human liver tumorigenesis, caspase-3 gene therapy led to a significant increase in apoptosis and shrinkage in tumour size when combined with other chemotherapeutic drugs [13, 32]. Caspase-8 expression has also been found to be impaired due to hypermethylation in several cancer cells. In small cell lung carcinomas, demethylation treatments have been shown to sensitise these cancer cells to drug-induced apoptosis [32, 53].

## 2.2. Anti-Bcl-2 therapy

The mitochondrial pathway is down-regulated by the anti-apoptotic Bcl-2 family proteins [19, 22, 40, 43, 60]. Drug-based therapy using anti-Bcl-2 small molecules has led to a significant

induction of apoptosis in several cancers. Flavipirodol, gossypol, depsipeptide, ABT-737, ABT-264, fenretinide, HA 14-1 and GX15-070 are some of the small molecules that inhibit BCl-2 by reducing their expression [41, 45, 59]. Small molecules with the ability to mimic pro-apoptotic or anti-apoptotic BH3-only Bcl-2 family proteins in order to induce apoptosis have also been designed [2, 41]. This class of drugs that imitate BH3-only pro-apoptotic and anti-apoptotic Bcl-2 family proteins is referred to as BH3-only mimetic drugs [2, 35]. ABT 737 is one example of the BH3-only mimetics that has been shown to inhibit expression of anti-apoptotic proteins such as Bcl-xL, Bcl-2 and Bcl-W; and is showing promising results in clinical trials [2, 59]. The first anti-Bcl-2 targeted drug to enter clinical trials in leukemic patients is known as oblimersen sodium [41, 59]. This Bcl-2 antisense has been shown to increase survival rates in chronic myeloid leukaemia patients when combined with chemotherapy [41, 59].

### 2.3. p53-based gene therapy

The loss of p53 function is a common feature in almost all human cancer including breast cancer [37, 43, 47]. Because of this, there is a lot of interest in targeting p53 for anticancer therapeutic drugs [7, 10, 16, 55]. The first biological approach which is now widely used in targeting p53 is gene delivery of wild-type p53 into tumour cells using adenoviral or retroviral techniques [28, 48]. p53-based gene therapy is however not effective on its own in killing cancer cells and for this reason combinational therapies involving other modes of treatments in the presence of p53 therapy are being investigated [10, 55, 59].

For example, it was discovered that concurrent treatment using adenoviral-mediated wild-type p53 injection with ionising therapy significantly reduces tumour size in cancers of prostate, brain and spine as well as head and neck [28, 59]. Elimination of p53-defective cells using synthetic viruses designed to infect and kill cancer cells is another breakthrough in p53-based gene therapy [28, 48, 59]. One example is the ONYX-015 drug, which is a genetically engineered adenovirus that has been modified to infect and lyse p53-deficient cells [28]. Genetic alterations that take place in p53 during tumorigenesis can trigger the immune responses in both T- and B-cells [10]. This provides yet another interesting platform for p53-based anticancer therapy, and a number of p53-based vaccines are currently undergoing clinical trials [10, 59].

### 2.4. Small molecule approach in p53-based drug therapy

In comparison to large biological drugs that are present with complex structures, small molecular drugs are organic compounds designed to be extremely low in molecular weight and are made up of well-defined chemical structures that enable them to pass through the cell membrane when taken orally. A further advantage of small molecule drugs over biologics is that they are stable, mostly non-immunogenic and it is easy to characterise their molecular composition and heterogeneity. The mode of action for small molecules relies on their binding to specific biopolymers such as proteins and nucleic acids and act as effectors to alter function or activity of the specific biopolymer.

In cancer, small molecules are used to restore mutated proteins back to their wild-type forms and induce activity of proteins responsible for elimination of tumorigenic cells [30]. In p53-based drug therapy, several small molecules that can restore the function of mutated p53 have been investigated. One example of a small molecule drug known is CP-31398; which has been shown to restore p53 function by intercalating with p53-bound DNA and destabilising the p53-DNA interaction [30]. Another small molecule called Phikan083, which is a derivative of carbazole, has been identified as one of the small molecules that has the ability to restore mutant p53 too. The most advanced of these small molecules are those that act by interrupting the p53-MDM2 interaction which is responsible for the inactivation of wild-type p53 [51, 52, 54]. These include the nutlins, tenovins and the MI-219 [52]. MDM2 acts as a negative regulator of p53 by binding to and inactivating the function of p53. This activity results in the loss of p53-mediated apoptosis in cancer cells, thus promoting carcinogenesis. While the MI-219 small molecular drugs are responsible for the destabilisation of the MDM2-p53 interactions in order to selectively induce apoptosis and inhibit apoptosis, nutlins disrupt the MSM2-p53 complex and selectively induce senescence [51, 52, 54].

### 2.5. siRNA-based p53 therapy

There are certain cancers with no mutations in p53 but in which non-mutated p53 might be down-regulated by certain p53 negative regulators [30]. In these cancers, development of specific siRNAs for silencing of the negative regulatory genes is often used to activate p53 [10, 30, 55]. MDM2 E3 ligase and the viral E6 protein are two extensively studied negative regulators of p53 that are associated with cancer progression [51, 52, 54].

Under normal cellular conditions, p53 tumour suppressor gene is kept under tight regulation by the MDM2-p53 auto-regulatory feedback loop [36, 51, 52]. In response to stress stimuli such as DNA damage or radiation, activated p53 interacts with genes responsible for the induction of cell cycle arrest or apoptosis (figure 1) [36]. During cancer development, the interaction between p53 and MDM2 mediates p53 interaction with the ring finger domain of the MDM2 ubiquitin ligase for degradation of the p53 tumour suppressor protein [54]. This event compromises the occurrence of cell cycle arrest and p53-mediated apoptosis and facilitates abnormal cell proliferation[52]. The use of MDM2-specific siRNA to disrupt the p53-MDM2 interaction in breast cancer cells has been shown to induce apoptosis, inhibit cell proliferation and lead to decreased tumour size [36, 51, 52, 54].

The E6 viral protein is another thoroughly studied p53 negative regulator in HPV (human papillomavirus)-related cancers such as anogenital, cervical, head and neck cancers. During HPV infection, E6 protein expression increases in order to facilitate HPV replication and viral integration into the host cell. The E6 protein achieves this outcome by using its E3 ligase Hect domain to bind to and degrade the cellular tumour suppressor proteins p53 and pRB, thus abrogating the host cells' potential to initiate cell cycle arrest and apoptosis. Therapeutic strategies to disrupt E6-p53 interactions in the form of antisense and siRNA application specific to E6 viral protein have received the most attention in HPV-related cancer therapeutics [10, 30, 55].

A third ubiquitous protein suspected to be yet another negative regulator of active p53 especially in breast cancer progression is known as retinoblastoma binding protein 6 (RBBP6)

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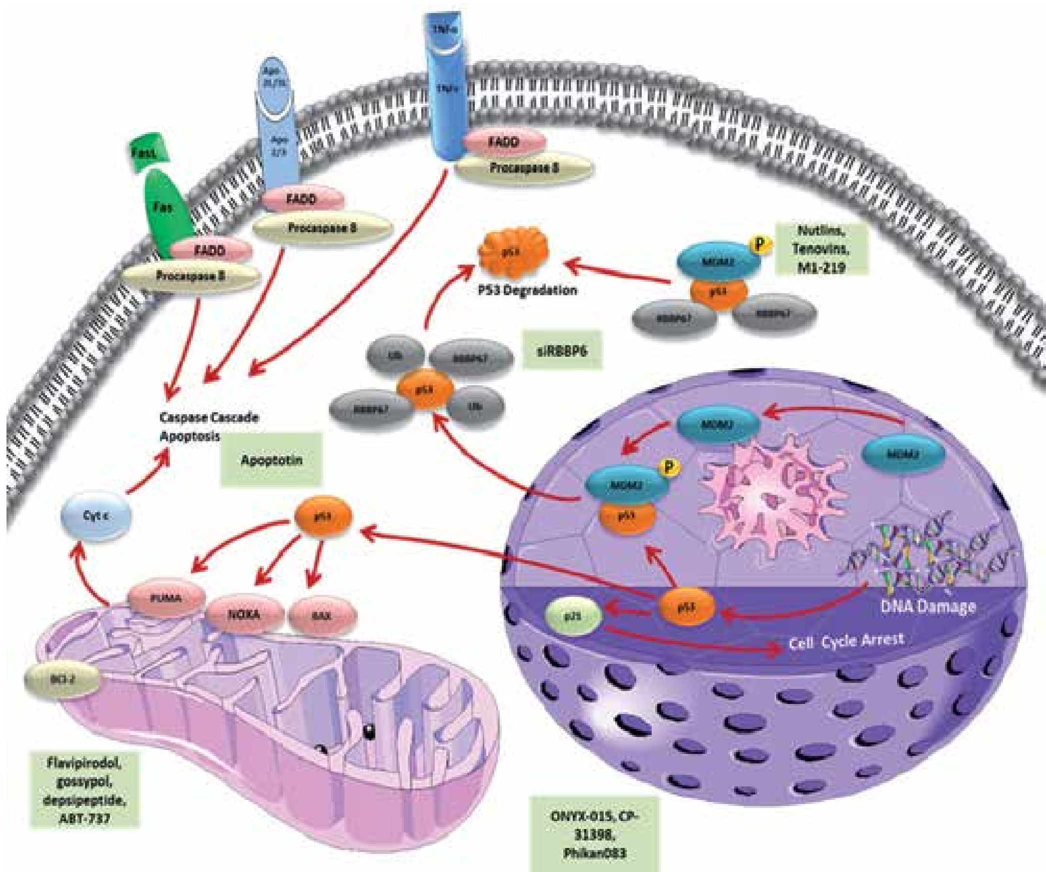


**Figure 1.** A simplified diagrammatic representation of the apoptotic signalling pathway and p53 negative regulation by MDM2 and another ubiquitous protein (RBBP6) suspected to be involved in p53 degradation. Current drugs that target different points of the apoptotic pathways are highlighted in light-green

[36]. RBBP6 is a 250kDa protein that has been shown to interact with and possibly lead to the degradation of p53 tumour suppressor gene since it possesses an E3 ligase activity [44, 49]. Its mRNA codes for a p53-binding domain as well as other domains known as DWNN domain, zinc finger domain and a ring finger domain, which are responsible for the ubiquitous nature of RBBP6 [44]. Besides the p53 domain, which is only present in human RBBP6, the above-mentioned domains are conserved in about all eukaryotic organisms such as humans, plants, protozoa, fungi, microsporidia and the single-celled parasite *Encephalitozoon cuniculi* [44]. RBBP6 is a spliced-associated protein and therefore exists in different other homologues known as PACT and P2P-R [44, 58].

A critical insight into the role played by RBBP6 in certain cancers via p53 has been elucidated [15]. Transfection of lung cancer cells with siRBBP6 led to a decrease in RBBP6 expression whereas sip53 transfection led to an increase in RBBP6 expression and, according to this study, RBBP6 may be involved in the degradation of p53 thereby enhancing abnormal cell prolifer-

ation [38]. In one study, it was demonstrated that down-regulation of the PACT homologue of RBBP6 in mice induces embryonic lethality with a consequent accumulation of p53 and a widespread apoptosis [33]. In addition to identifying PACT as a negative regulator of p53, further discoveries suggest that PACT knockdown enhances p53-Hdm2 interaction thus reducing p53 poly-ubiquitination by RBBP6 [33].

In recent studies, it was found that silencing RBBP6 gene in MCF-7 and CAMA-1 cells led to p53 up-regulation and sensitised the breast cancer cells to apoptosis induction [36]. Concurrent treatment of these cells with apoptosis-inducing agents, camptothecin or staurosporine, further increased apoptosis induction [36]. Furthermore, up-regulation of bax as a result of the co-treatment provided early insights into the possible mechanism behind the observed apoptosis [36]. Taken together, it is suspected that RBBP6 silencing may be responsible for the identified p53 up-regulation in breast cancer and other cancers and that the observed apoptosis is more likely p53-dependent; however, further *in vivo* investigations would validate these observations.

## 3. Conclusions

Taken all together, it is evident that anticancer therapeutics primarily depend on apoptosis pathway activation in breast cancer and several other cancers. However, a few milestones still need to be reached with regard to this novel anti-tumour molecular approach. For example, most of the experimentally studied apoptosis-inducing regimens in breast cancer cells have not reached the clinical stages. Another important factor that needs to be addressed in apoptosis-targeted therapy is to determine whether the observed cytotoxicity of breast cancer cells in experimental settings is comparable in clinical settings. Moreover, understanding tumour biology of individual cancer patients can help select therapeutic interventions that are highly specific to a presented tumour. Nonetheless, the link between apoptosis and tumorigenesis has been thoroughly investigated in breast cancer and has led to lots of promising strategies that attempt to eradicate cancer cells by targeting the apoptosis signalling pathway.

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Chapter 11

# Between Armour and Weapons — Cell Death Mechanisms in Trypanosomatid Parasites

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Additional information is available at the end of the chapter

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**Abstract**

Among the pathogenic protozoa, trypanosomatids stand out due to their medical and economic impact, especially for low-income populations in tropical countries. Together, sleeping sickness, Chagas disease and leishmaniasis affect millions of humans and animals worldwide, yet are neglected by the pharmaceutical industry. The current drugs for trypanosomatid infections are limited and unsatisfactory, with severe side effects leading to reduced quality of life and, in several instances, to the abandonment of treatment. An intense search for alternative compounds has been performed, aiming at specific parasite targets by cellular, molecular and biochemical approaches. One interesting strategy could be interference with the protozoan cell death pathways. However, these pathways are poorly understood in unicellular eukaryotes, with the controversial existence and uncertain biological relevance of programmed cell death (PCD). This chapter will discuss apoptosis-like and autophagic cell death and necrosis in *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* sp. and the possible implications of these pathways for the parasite life cycle and infection persistence. It will also revisit the genomic and proteomic metadata of these trypanosomatids in the literature to rebuild the map of cell death proteins expressed under different conditions. The interaction of leading candidates with parasite-specific molecules, especially with enzymes that regulate key steps in the cell death process, is a rational and attractive alternative for drug development for these neglected diseases.

**Keywords:** Cell death, apoptosis-like, autophagy, necrosis, *Leishmania* sp, *T. cruzi*, *T. brucei*

## 1. Introduction

Neglected tropical diseases (NTDs) are a group of the seventeen mostly life-threatening infections, which affect more than a billion people worldwide. They affect poor populations,

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often in underdeveloped and developing countries (low-income countries) [1]. Among NTDs, infections caused by the so-called "protozoan" parasites, such as African trypanosomiasis, Chagas disease and leishmaniasis, are responsible for a high annual death toll among the poor populations of tropical countries. New safe and affordable medicines are urgently needed. These diseases all present therapeutic difficulties by developing resistance to existing therapies and/or by toxic side effects.

### 1.1. Neglected tropical diseases and trypanosomatids

#### *1.1.1. Sleeping sickness*

Human African trypanosomiasis (HAT), or sleeping sickness, is caused by extracellular protozoa belonging to the genus *Trypanosoma* and the species *T. brucei*. Two subspecies of *T. brucei* cause diseases with different epidemiological and clinical patterns: *T. b. gambiense*, a chronic disease present in western and central Africa accounting for 98% of the cases, and *T. b. rhodesiense*, an acute zoonosis located in eastern and southern Africa that occasionally infects humans. In 2001, WHO launched a major initiative to reinforce disease control and surveillance. After 10 years, the number of new cases of HAT decreased by 73.4%. Presently, the estimated the number of cases is 30, 000, and 70 million people are at risk [2, 3]. HAT clinically evolves in two stages. In the first stage, parasites are found in the lymphatic system and bloodstream. After a variable period of time, which is much shorter for the rhodesiense form, the second stage begins, with the parasites penetrating the blood-brain barrier and invading the central nervous system, leading to progressive neurological damage [4]. HAT is usually fatal if left untreated. Rhodesiense HAT usually progresses to death within six months, while gambiense HAT has a more chronic progressive course with an average duration of almost three years [5].

*T. brucei* is transmitted by the tsetse fly Glossina spp when it takes a blood meal. Non-dividing metacyclic forms enter the bloodstream of the mammalian host and differentiate into a rapidly dividing slender form able to evade antibody responses through antigenic variation [6]. Most of these forms undergo cell cycle arrest and develop into short-stumpy forms. When the tsetse fly bites an infected host, only the short-stumpy parasites survive in the insect's midgut and develop into a procyclic form, which undergoes multiple developmental phases on its way to the salivary gland, finally culminating in the infective metacyclic form [7, 8].

The drug of choice for treatment depends on the infecting species and the stage of infection. In early stages, *T. b. gambiense* and *T. b. rhodesiense* infections can be treated with pentamidine and suramin, respectively [9]. If the disease has progressed, treatment relies on melarsoprol or eflornithine. Melarsoprol, an arsenical drug, is extremely toxic. Eflornithine is less toxic, but is expensive, and has a difficult administration than melarsoprol and lacks efficacy against *T. b. rhodesiense* [2]. Since 2001, this drug has been combined with nifurtimox (NECT) for first-line treatment for CNS-stage *T. b. gambiense* HAT. It is the most recent breakthrough in anti-trypanosomiasis drug research and was added to the World Health Organisation's list of essential medicines in 2009. A major problem related to the treatment of HAT is the development of resistance to melarsoprol and the other drugs [10].

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#### *1.1.2. Chagas disease*

Chagas disease is caused by the intracellular obligatory parasite *Trypanosoma cruzi* and affects approximately eight million individuals in Latin America [11]. The transmission of this disease occurs through the faeces of sucking triatominae insects, blood transfusions, organ transplantation, oral contamination, laboratory accidents and congenital routes [12, 13]. Current major concerns are the outbreaks of acute Chagas disease associated with the ingestion of contaminated food and its emergence in non-endemic areas, such as North America and Europe, due to the immigration of infected individuals [14-16]. This disease is characterised by two clinical phases. The acute phase appears shortly after infection and is defined by patent parasitaemia. If left untreated, symptomatic chronic disease develops in about one-third of individuals after a long latent period (10-30 years), which is known as the indeterminate form. The main clinical manifestations of Chagas disease include digestive and/or cardiac alterations. The chronic cardiac form of the disease is the most significant clinical manifestation. Consequences include dilated cardiomyopathy, congestive heart failure, arrhythmias, cardioembolism and stroke [17].

The life cycle of *T. cruzi* involves four major developmental stages during its passage through vertebrate and invertebrate hosts [18]. The infective stage of the parasite, the metacyclic trypomastigote, enters the mammalian host from insect faeces through wound openings or mucous membranes. In the mammalian host, the metacyclic trypomastigote differentiates into the amastigote form. After several rounds of replication in the host cells, the amastigote differentiates into the bloodstream trypomastigote, which can enter new cells and perpetuate the infection. When the insect bites an infected host, the bloodstream trypomastigote differentiates into the replicative epimastigote that lives in the insect's gut. Finally, in the rectum of the insect, the epimastigote differentiates into the infective metacyclic trypomastigote, which is ready to infect its host again.

The available chemotherapy for this illness includes two nitroheterocyclic agents, nifurtimox and benznidazole, which are effective against acute infections, but show poor activity in the late chronic phase, with severe collateral effects and limited efficacy against different parasitic isolates. These drawbacks justify the urgent need to identify better drugs to treat chagasic patients, and several new compounds are currently in preclinical development involving *in vitro* parasite phenotype screens and target-based drug discovery [19-21]. Recently, clinical trials with the azoles posaconazole and E1224 (ravuconazole prodrug) led to higher percentages of treatment failure in chronic patients than benznidazole [22, 23], suggesting their potential use in combination therapy [24].

#### *1.1.3. Leishmaniasis*

Leishmaniasis, which is caused by different species of *Leishmania,* is a vector-borne disease, with an estimated 12 million cases worldwide. Infection is caused by the bite of infected female sand flies of the genera *Phlebotomus* (Europe, Asia, Africa) and *Lutzomyia* (America) [25]. *Leishmania* parasites live a digenetic life cycle as either a promastigote flagellar or an amastigote form. The type of clinical manifestation depends on the infecting species and host factors, such as general health and genetic and immune constitution [26]. It is a disease complex with three

clinical manifestations, visceral (VL, kala-azar), cutaneous (CL) and muco-cutaneous (MCL), which arise from parasite replication in the mononuclear phagocyte system, dermis and naso-oropharyngeal mucosa, respectively [27]. Some post-treated *L. donovani*-infected patients develop the diffuse cutaneous form named post-kala-azar dermal leishmaniasis (PKDL) [28, 29]. VL, after initial skin lesions, takes 2-8 months to develop gross inflammatory reactions within the viscera (liver and spleen in particular) and is usually fatal unless treated. CL manifests as an open sore at the site of the insect bite and will frequently self-heal, leaving a scar. The diffuse form of CL is more problematic, causing lepromatous type lesions disseminated across the skin that can be difficult to heal. The MCL form, endemic in parts of Latin America, starts with skin sores that spread to the mucosal membranes of the face. Profound inflammatory damage can lead to the erosion of the nostrils and mouth in particular [29].

In the *Leishmania* life cycle, there are two principal parasite forms: amastigotes and motile promastigotes. In the alimentary tract of the insect vector, the parasite exists as multiplicative, non-infective procyclic promastigotes and non-multiplicative, infective metacyclic promastigotes [30]. Upon injection into the mammalian host, promastigotes are taken up by macrophages where the metacyclic forms differentiate into small multiplicative, non-motile amastigotes that live in a lysosomal compartment known as the parasitophorous vacuole [31]. These developmental forms are distinguished by their nutritional requirements, their growth rate and ability to divide, the regulated expression of their surface molecules, and their morphology. Metacyclic promastigotes are pre-adapted for survival in the mammalian host, as they are complement-resistant. Amastigotes are intracellular, non-motile forms that have adapted to the low pH of this compartment and have an adapted energy metabolism.

The current drugs are highly toxic, resistance is common and compliance of patients to treatment is low, as the treatment is long and the drug price is high. Although recent initiatives have improved the antileishmanial drug arsenal by combining current medicines or using new formulations of old ones, none are ideal for treatment due to their high toxicity, resistance issues, prohibitive prices, long treatment length and need of intravenous administration [32-34]. Pentavalent antimonials (glucantime and pentostan) are first-line drugs for both VL and CL. However, they present several limitations, including variable efficacy, need for daily injectable administration for approximately one month, and severe side effects. Many patients are unable to complete the treatment, increasing the risk of drug resistance development. Amphotericin B is a systemic antifungal that is used as a second-line drug for VL. It is highly toxic, requiring careful and slow intravenous administration. Lipid formulations of amphotericin B have been developed to improve its bioavailability and pharmacokinetic properties, reducing toxicity [35]. Miltefosine is the most recent antileishmanial drug on the market and the first effective oral treatment against VL [36]. However, it has common gastrointestinal side effects and is also limited by its relatively high cost [34], potential teratogenicity and growing concerns in relation to increases in clinical isolate susceptibility [37]. Paromomycin is an aminoglycoside antibiotic that is used in topical treatment for CL and as a parenteral drug for VL. Pentamidine was used as a second-line drug in antimony-resistant VL treatment. However, its high toxicity combined with decreased efficacy led to the abandonment of this drug to treat VL in India, but it is valuable for combined therapies [38].

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## 2. Cell death: State of art

As used for whole organisms, the term death is employed to describe a sequence of events culminating in the breakdown of all biological functions. However, more than one century after the first citation [39], cell death still represents a crucial gap in our understanding of cellular physiology. It can be triggered by natural processes or induced by extrinsic factors (exposure to chemicals or physical stresses). The consequent tissue injury usually leads to a state of disease [40]. On the other hand, many studies pointed to cell death playing a fundamental role in the physiology of multicellular organisms, especially in processes such as metamorphosis and embryogenesis [41]. In this context, in 1964, the term programmed cell death (PCD) was created, proposing a sequence of well-controlled steps regulating a non-accidental cell death process in the absence of an inflammatory response [42]. Currently, it is known that distinct death mechanisms and phenotypes participate in PCD, with apoptosis and autophagy being the most prominent [43].

### 2.1. Apoptosis

The apoptotic pathway was first described in the early 1970s as a fundamental step for proper embryo development [44]. This process is crucial during tissue development, especially in immune response regulation and removal of infected or damaged cells [45, 46]. Apoptosis is involved not only in growth regulation in multicellular organisms [47, 48] but also in their defence against viral, bacterial or parasitic infections [49-53] and even against cancer development [54-57]. The removal of non-functional cells by the apoptotic pathway is efficient and prevents the inflammatory response [58].

During apoptosis in multicellular organisms, the cell activates death machinery that culminates in chromosomal condensation and nuclear DNA fragmentation [59, 60]. Biochemically, apoptosis is orchestrated by the activation of a family of cysteine proteases, named caspases, that are activated by extrinsic and intrinsic factors [45, 46]. The extrinsic pathway is activated by the interaction of death ligands with their respective cell surface receptor (i.e., FasL/Fas, TNF-$\alpha$/TNFR) [61-63]. Such binding triggers the cleavage of procaspase 8 into active caspase 8, which cleaves procaspase 3. Executioner caspase 3 activates endonuclease G (EndoG), starting the characteristic DNA fragmentation, a distinctive marker of apoptosis [63-65]. On the other hand, the intrinsic pathway can be triggered by two distinct mechanisms with mitochondrion or endoplasmic reticulum (ER) dependency. In the mitochondrial pathway, activation occurs by membrane permeabilization, releasing cytochrome c, apoptosis induction factor (AIF), EndoG and regulators of the B-cell lymphoma 2 (Bcl2) protein family into the cytosol. In the cytosol, the apoptosome is formed by the interaction of released cytochrome c with apoptotic protease activating factor 1 (APAF-1) and procaspase 9, activating caspase 9, which subsequently activates the effector caspase 3 [66-70]. The ER pathway is mainly caspase 12-dependent and occurs in this organelle during stress conditions. Because this pathway was described in the mouse and humans lack functional caspase 12, the relevance of ER-mediated apoptosis is still debatable [71-73].

Undoubtedly, the caspase cascade represents a central point in the apoptotic process. Its regulation is well-controlled by pro- and anti-apoptotic molecules from the Bcl-2 family [74]. The apoptotic morphological and biochemical phenotypes include cell shrinkage, membrane blebbing (formation of apoptotic bodies), chromatin condensation and typical internucleosomal DNA fragmentation, externalization of phosphatidylserine (PS), loss of mitochondrial membrane potential ($\Delta\Psi m$), and target protein degradation by caspase activation [75-79]. The characterization of apoptosis is experimentally based on the detection of apoptotic markers. The loss of $\Delta\Psi m$ (labelling with rhodamine 123 derivatives, such as TMRE), PS exposure (binding to labelled annexin V), chromatin condensation (DAPI labelling) and DNA fragmentation (TUNEL technique) are usually quantified by fluorescence microscopy or flow cytometry. DNA fragmentation can also be assessed by agarose gel electrophoresis, presenting a laddering pattern that represents internucleosomal cleavage. Analysis of caspase activity using labelled specific substrates and/or inhibitors can be performed by immunotechniques such as ELISA [80].

### 2.2. Autophagy

In the 1950s, acidic organelles involved in the intracellular degradation of macromolecules were described and termed lysosomes by Dr. Christian de Duve. In a subsequent study [81], he proposed the term autophagy for a self-degrading process [82]. Currently, the autophagic pathway is considered to be the main cellular mechanism for the degradation of non-functional organelles and/or macromolecules and is fundamental for homeostasis in eukaryotic cells [83]. In other words, autophagy is a housekeeping self-digestion mechanism that is crucial for cellular turnover and recycling and occurs by the engulfment of cytosolic portions containing material that should be degraded. Degradation starts immediately after the fusion of autophagosomes to lysosomes in an organelle named the autophagolysosome [84, 85].

In multicellular organisms, autophagy is involved in many physiological situations, including development, cell growth and cell differentiation. Autophagy sustains cell survival under 'extracellular stress', such as nutrient starvation, hypoxia, acidic pH and high temperature. It acts as a housekeeping device under 'intracellular stress' by removing damaged or redundant cytoplasmic components, including organelles [86]. Increased autophagic activity is observed in pathological states and in host defences against pathogens [87-92]. Despite the relevant role of autophagy for the maintenance of the regular cell cycle, prolonged starvation periods or other strong autophagic stimuli induce a cellular misbalance and promote autophagic cell death [93, 94].

The autophagic molecular machinery was first assessed in the yeast model *Saccharomyces cerevisiae*, and 30 proteins, called Atgs (AuTophaGy-related), were described and associated with different steps of the pathway [95]. Atg orthologues were identified in all eukaryotes, with Atg8 (LC3 in mammals) being one of the most studied [82]. Autophagy can be a selective or non-selective process, degrading specific or random cellular components. Examples of selective routes are mitophagy, pexophagy or reticulophagy, in which mitochondria (or part of the organelle), peroxisomes and ER are degraded, respectively [82].

Additionally, there are three types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). The most common is macroautophagy, a process that involves the engulfment of cytosolic portions by a double membrane structure called the phagophore. The double-membrane vesicle formed from phagophore engulfment is named the autophagosome and is directed to lysosomes for degradation by lysosomal hydrolases. These steps are regulated by Atgs [92, 96-98]. The chronological events related to macroautophagy are (a) autophagic induction; (b) cargo selection; (c) phagophore elongation; (d) autophagosome formation; (e) fusion to lysosomes; and (f) cargo degradation [99]. The early steps in this process depend on the serine/threonine protein kinase TOR (target of rapamycin), which is essential for autophagic regulation. TOR complexes 1 and 2 work as sensors of nutritional availability (especially amino acids). The autophagic enzyme Atg6 (Beclin 1 in mammals) is a phosphatidylinositol 3-kinase (PI-3K) and shares its signalling function with other cellular pathways. For autophagy, these kinases present a critical role for autophagosome formation [82].

In contrast, there are no autophagosomes in the microautophagic pathway. Invagination of the lysosomal membrane occurs, resulting in a single-membrane small vesicle inside the lysosomes that will be degraded. Interestingly, both macro- and microautophagy could be selective or non-selective processes. Indeed, CMA appears to be the most selective type of autophagy. The proteins that will be degraded contain pentapeptide motifs (KFERQ, QREFK or VDKFQ), the binding sites of a cytosolic chaperone. Such a chaperone-substrate complex binds to a LAMP-2A receptor in the lysosomal membrane, promoting receptor dimerization. A membrane channel is formed, and the specific protein reaches the lysosomal lumen to be degraded [82, 100].

For many years, electron microscopy was the only tool available for the identification of autophagic morphological features, especially the presence of double-membrane vesicles (autophagosomes). In the last 20 years, advances in the molecular description of autophagy allowed the detection, localization and quantification of Atgs by molecular, biochemical and morphological approaches. Currently, the gold-standard method to monitor autophagy is Atg8/LC3 detection by different techniques: (a) Western blotting (presence of two isoforms); (b) confocal or fluorescence microscopy (identification of LC3 puncta); (c) knock down or knock out (deletion and analysis of the phenotype); and (d) pharmacological induction/inhibition (rapamycin and/or PI-3K inhibitors). These techniques can also be employed *in vitro* or *in vivo* for other Atgs, indicating autophagic activity [101].

### 2.3. Necrosis

Necrosis is a term that is extensively employed as synonymous with cell death. In the Greek aetiology, it signifies the "stage of dying". In this death type, strong cellular damage occurs caused by external stimuli (drugs, infection, mechanical trauma), promoting the random degradation of the whole cell, with plasma membrane disruption. Necrosis is defined as an accidental cell death process, differing from PCD (especially apoptosis) [102]. One of the main differences between apoptosis and necrosis is the induction of the inflammatory response in the latter. The release of intracellular material into the extracellular environment during

necrotic cell death triggers intense inflammation in the surrounding cells and tissues [103]. Classical necrotic features are the loss of plasma membrane integrity, cytosolic vacuolization, disruption of calcium homeostasis, general degradation by lysosomal hydrolases and induction of the inflammatory response.

Necrosis can also be a regulated process. Necroptosis is a programmed and non-accidental death pathway. Surprisingly, the activation of this pathway can occur by TNF-$\alpha$ or FasL, classical apoptotic ligands. Necroptosis depends on the participation of the receptor-interacting protein kinases 1 and 3 (RIPK1 and RIPK3), which are kinases that regulate this pathway. RIPK1 is pharmacologically inhibited by a small molecule named necrostatin-1 (Nec-1) [104-106].

### 2.4. Others

In addition to apoptosis, autophagy and necrosis (accidental or not), other non-canonical death styles can take place in eukaryotic cells. In an inflammatory context, pyroptosis and NETosis are prominent. Pyroptosis, primarily observed in macrophages after bacterial infection, is caspase 1-dependent. This caspase promotes an increase in the inflammatory cytokine levels (IL-1β and IL-18) and the formation of plasma membrane pores, leading to the release of cellular material to the extracellular matrix. The main difference between pyroptosis and apoptosis is the participation of caspase 1, which is only involved in the pyroptotic death pathway, a proinflammatory PCD [106-108]. Another type of cell death that plays a crucial role in the innate immune response is the neutrophil extracellular trap (NETosis), where neutrophilic death leads to the release of a neutrophil DNA network coated with histones and elastase to the extracellular environment to capture pathogens. However, the direct antimicrobial effect of the NETs is still controversial [109, 110]. Currently, DNA release has also been described in other immune cells, such as eosinophils, basophils, macrophages and mast cells, but its precise role deserves further analysis [110-114].

Other cell death types not involved in inflammation have been characterized. Ferroptosis is iron-dependent cell death that has been identified in some mammalian cells and involves oxidative stress induced by a small molecule named erastin, which is inhibited by ferrostatin 1. Despite that lack of complete understanding of the erastin mechanism, the $X_C$–Cys/Glu antiporter system is inhibited in ferroptosis, leading to a misbalance of these amino acids inside the cell [106, 115]. Additionally, there is another non-canonical cell death pathway in cancer cells (*in vitro* and *in vivo* models) called autoschizis, which involves oxidative stress induced by treatment with ascorbate and menadione. Autoschizic cell death presents remarkable morphological evidence, with electron microscopy as the best technique for its identification. Among the autoschizic features are cell shrinkage, extrusion of large portions of the cytosol (without any organelles), random DNA fragmentation and the subsequent deterioration of all cellular structures [116, 117]. Interestingly, annexin V (AV) and propidium iodide (PI) assays (gold standards for apoptosis detection in mammals) of cells treated with ascorbate and menadione demonstrate high percentages of AV-/PI+ cells [117], which are not discussed in almost all apoptotic studies, suggesting that these membrane shedding events could occur in a large variety of cell models. Table 1 summarizes the main types of cell death discussed herein.

| Cell death | Features | References |
|---|---|---|
| apoptosis | cell shrinkage<br>membrane blebbing<br>DNA fragmentation<br>externalization of PS<br>activity of caspases<br>regulation by Bcl-2 family proteins<br>loss of $\Delta\Psi$<br>release of cytochrome c<br>no inflammatory response | [44, 76, 78] |
| autophagy | presence of autophagosomes<br>participation of Atgs<br>regulation by PI-3K and TORC<br>degradation by lysosomes<br>presence of KFERQ, QREFK or VDKFQ motifs in the protein to be degraded (only in CMA) | [82, 101] |
| necrosis | disruption of plasma membrane<br>cytoplasmic vacuolization<br>imbalance of Ca2+ homeostasis<br>release of lysosomal enzymes<br>induction of inflammatory response | [102, 103] |
| necroptosis | participation of RIP1 and RIP3<br>inhibition by Nec-1 | [104, 106] |
| pyroptosis | participation of caspase 1<br>increase in IL-1$\beta$ and IL-18 levels<br>induction of inflammatory response | [106-108] |
| NETosis | formation of NETs<br>participation of elastase and histones<br>occurrence in neutrophils, macrophages, mast cells, eosinophils and basophils | [109, 110] |
| ferroptosis | participation of iron<br>presence of oxidative stress<br>induction by erastin<br>blockage of $X_C$–Cys/Glu antiporter system<br>inhibition by ferrostatin 1 | [106, 115] |
| autoschizis | cell shrinkage<br>random DNA fragmentation<br>extrusion of large cytosolic portions (without organelles)<br>degradation of cellular components<br>increase in the AV-/PI+ population | [116, 117] |

**Table 1.** Types of cell death

## 3. Cell death in trypanosomatids: An overview

The term PCD was employed for decades to exclusively describe cell death in metazoans and its involvement in embryogenesis and maintenance of homeostasis. Indeed, the relevance of PCD for lower eukaryotes is unclear. In an evolutionary scenario, these regulated processes could allow clonal selection in the parasite population, guaranteeing the propagation of identical genetic information even in adverse environmental conditions. However, differences in the cell death mechanisms observed between metazoans and protozoans must be considered [78, 118]. In the following sections, we will discuss the role of different death styles described in pathogenic trypanosomatids.

### 3.1. Apoptosis-like

In trypanosomatids, the first PCD report was published in 1995 by Ameisen and coworkers describing apoptotic characteristics (DNA fragmentation and cytoplasmic and nuclear morphological alterations) in *T. cruzi* epimastigotes during differentiation to trypomastigotes [119]. In the last two decades, a variety of stimuli were reported to induce the appearance of the apoptotic phenotype in this parasite, including exposure to fresh human serum (FHS), heat shock and drugs [76, 119-128]. Curiously, the apoptosis-like phenotype was also associated with the regulation of the *T. cruzi* life cycle [129]. These cell death phenotypes in pathogenic trypanosomatids have been characterized by the use of classical apoptotic markers (see item 2.1) [76, 79, 118, 123, 130-133]. Among the apoptotic hallmarks identified, we found (a) loss of $\Delta\Psi m$, (b) cytochrome c release, (c) PS externalization, and (d) abnormal DNA condensation and fragmentation [76, 119, 129, 130, 134] (Table 3, Figure 1).

In *Leishmania* sp., apoptotic features (nuclear condensation, DNA fragmentation, cell shrinkage, loss of $\Delta\Psi m$, and release of cytochrome c) were also observed in stress conditions induced by heat, starvation, oxidative agents and drugs [118, 134, 136-139, 135]. *L. donovani*, *L. major* and *L. mexicana* stationary phase promastigotes and axenic amastigotes exhibited DNA fragmentation with a laddering electrophoretic profile, suggesting oligonucleosomal cleavage. These data were corroborated by the description of a non-canonical, $Ca^{2+}$- and $Mg^{2+}$-independent 45-59 kDa endonuclease [76, 136, 140].

As in other pathogenic trypanosomatids, apoptotic features were also identified in *T. brucei* under non-physiological conditions, such as incubation with drugs, cytokines or ROS [129, 133, 141-143]. Interestingly, the gene for prohibitin and the receptor for activated protein kinase C have been correlated with the apoptotic process, suggesting convergence between these pathways in protozoa and mammals (Table 2) [129]. Despite several reports about caspase-like activity in trypanosomatids [75, 134, 136, 144], the exact role of these proteases in protozoa is not clear. Metacaspases are structurally similar to mammalian orthologues, but their catalytic activity on caspase substrates is quite controversial [145-147]. Despite their presence in *T. cruzi*, *T. brucei* and *Leishmania* sp., only *L. major* metacaspase shows *in vitro* self-proteolytic activity (Table 2) [146]. In fact, the participation of metacaspases cleaving vital substrates in the cell death cascade has not yet been described [148, 149]. Surprisingly, experimental

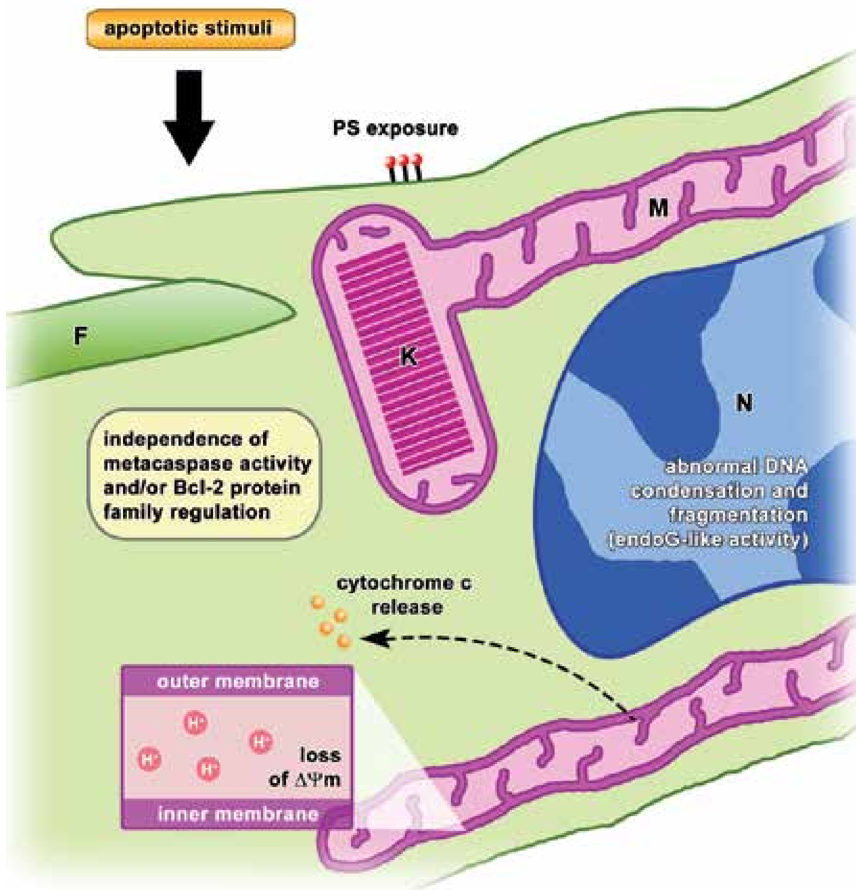


**Figure 1.** Schematic representation of apoptosis-like PCD in pathogenic trypanosomatids. Apoptotic stimuli induce loss of ΔΨm, release of mitochondrial cytochrome c to the cytosol, PS externalization and DNA fragmentation by EndoG activity. Apoptotic regulators from the Bcl-2 family were not found until now, and the role of metacaspases is controversial, suggesting that apoptosis-like PCD in trypanosomatids is a caspase-like- and Bcl-2-independent pathway. N: nucleus; M: mitochondrion; K: kinetoplast; F: flagellum.

evidence pointed to the involvement of these proteases in cell cycle control and metacyclogenesis, not in death [145, 150-153].

In unicellular organisms, the mitochondrion is a central organelle in cell death pathways, leading to ROS production [125]. In *T. brucei* procyclic forms, mitochondrial $Ca^{2+}$ influx misbalance culminates in ROS generation [154]. Additionally, prostaglandin D2-induced ROS production in both the bloodstream and procyclic forms led to the labelling of different apoptotic markers, with the death phenotype reverted by oxidative scavengers, such as N-acetyl cysteine [130, 155, 156]. In *L. donovani*, hydrogen peroxide induced classical apoptotic features (DNA fragmentation, loss of ΔΨm and caspase-like activity). This phenotype was partially reverted by caspase inhibitors [134, 137]. Oxidative stress plays a crucial role not only in apoptosis-like PCD but also in autophagy and necrosis, as we will discuss later [78, 157].

| Molecule | Organism | References |
|---|---|---|
| Prohibitin<br>RACK | *T. brucei* | [129] |
| Elongation factor 1 ∝ | *T. cruzi* | [161] |
| Metacaspases 1 | *L. donovani*<br>*T. brucei* | [147, 162, 163] |
| Metacaspases 2 | *L. donovani*<br>*T. brucei* | [145, 147, 162] |
| Metacaspases 3 | *T. cruzi*<br>*T. brucei* | [145, 150, 153, 162] |
| Metacaspases 4 | *T. brucei* | [162, 164] |
| Metacaspases 5 | *T. cruzi*<br>*T. brucei*<br>*L. major* | [145, 150, 162, 165] |
| Metacaspase Z-DEVD-FMK -sensitive | *T. cruzi*<br>*L. donovani* | [124, 134, 136] |
| Endonuclease G | *L. major*<br>*T. brucei*<br>*L. infantum*<br>*L. donovani* | [132, 158, 166] |
| LdFEN-1<br>LdTatD-like nuclease | *L. donovani* | [158] |

**Table 2.** Apoptotic molecules described in pathogenic trypanosomatids

The participation of EndoG-like in mitochondrial-mediated cell death has been reported, but the process is metacaspase-independent (Table 2) [132, 158, 159]. *L. infantum* submitted to heat stress also presents an apoptotic pattern, but without caspase-like activity, which was partially reversed by the expression of the anti-apoptotic mammalian gene Bcl-XL [160]. On the other hand, the overexpression of mammalian anti-apoptotic Bcl-2 in *T. brucei* caused no reversion of the mitochondrial damage induced by ROS [154]. However, members of the Bcl-2 protein family have not been described in trypanosomatids [129]. More studies regarding the regulation steps of apoptosis-like processes in trypanosomatids need to be performed.

### 3.2. Autophagy

Almost forty years ago, the first morphological autophagic evidence was described in trypanosomatids by electron microscopy of *T. brucei* [170]. In the last four decades, many studies have described recurrent autophagosome formation (initially named autophagic vacuoles), multivesicular bodies as well as myelin-like structures in pathogenic trypanosomatids treated

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with different classes of drugs (Figure 2) [169, 168, 171-178]. Such autophagosomes showed distinct levels of degradation depending on the degree of cellular structure damage inside the organelle. Myelin-like structures are one of the most frequent ultrastructural alterations detected in drug-treated parasites and are suggestive of the cellular recycling of damaged structures. Currently, it is postulated that myelin-like structures are phagophores (or pre-autophagosomal structures, PAS), an early step in the formation of doubled-membrane autophagosomes (Table 3). In *T. cruzi*, ER profiles were reported as the main origin of phagophores (Figure 2). These profiles usually surround a pre-lysosomal compartment, named the reservosome, suggesting the participation of this organelle in autophagolysosome formation in epimastigote forms [82, 178].

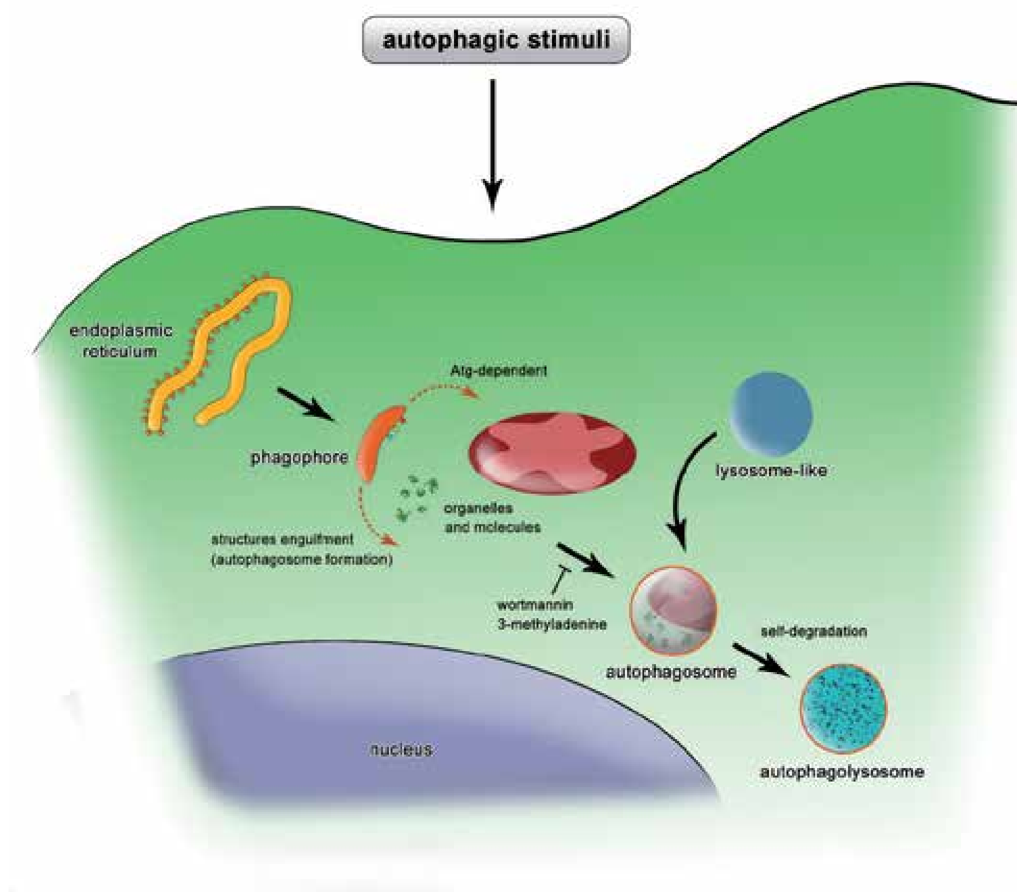


**Figure 2.** Schematic representation of autophagy in pathogenic trypanosomatids. Autophagic stimuli induce the formation of phagophores from ER profiles. The phagophore engulfs organelles and molecules, generating autophagosomes. Targeting and engulfment are Atg-dependent processes. These autophagosomes fused with lysosomes generate autophagolysosomes. Continuous autophagic stimuli lead to autophagic cell death, which is inhibited by the pre-treatment of the parasite with autophagic inhibitors (wortmannin or 3-methyladenine).

| Cell death | Features | Organism | References |
|---|---|---|---|
| apoptosis-like | cell shrinkage<br>membrane blebbing<br>DNA fragmentation<br>PS exposure<br>loss of the ΔΨm<br>release of cytochrome c | *T. cruzi*<br>*T. brucei*<br>*Leishmania* sp. | [76, 118, 119, 130] |
| autophagy | presence of autophagosomes-like<br>Golgi and/or ER profiles surrounding organelles<br>detection of Atg8 and Atg4 | *T. cruzi*<br>*T. brucei*<br>*Leishmania* sp. | [82, 92, 167] |
| necrosis | cytosolic vacuolization<br>plasma membrane disruption | *T. cruzi*<br>*T. brucei*<br>*Leishmania* sp. | [149, 168, 169] |

**Table 3.** Types of cell death described in pathogenic trypanosomatids

In the last few years, a functional autophagic pathway was characterized in trypanosomatids and ATG homologues were identified. However, almost half of the yeast Atgs are lacking in these protozoa [167, 179-181]. Currently, in trypanosomatids, twenty autophagic genes have been found to be involved in all of the steps, from vesicle expansion and completion to degradation (Figure 2) [167]. Bioinformatic approaches revealed all four genes of the Atg8 conjugation system (Atg3, Atg4, Atg7 and Atg8). Atg8 is well-characterized in *T. cruzi*, *T. brucei* and *Leishmania* sp. and is located in autophagosomes, as observed in yeast and mammals. Atg8 has four isoforms (Atg8, Atg8A, Atg8B and Atg8C) that are processed by two isoforms of Atg4 (Atg4.1 and Atg4.2) [92, 181-185] (Table 3). On the other hand, the Atg12 conjugation system has poor sequence similarity in trypanosomatids, and Atg5, Atg10 and Atg12 sequences are lacking [167, 180]. Pathogenic trypanosomatids have two TOR kinases (TOR1 and TOR2) that form their respective complexes, TORC1 and TORC2 (Table 4). These two complexes show a distinct molecular behaviour, subcellular localization and susceptibility to rapamycin [186, 187]. The treatment of *T. brucei* bloodstream forms with rapamycin led to cell cycle arrest and an increase in the number of autophagosomes due to TORC2 inhibition. However, rapamycin had no effect on the parasite TORC1, suggesting another function for this complex [186, 188].

In addition to the recycling function, autophagy plays a fundamental role in parasite differentiation and survival, mitochondrial function and homeostasis of phospholipids [92, 182, 189, 190]. In metacyclogenesis, the autophagic pathway is triggered by nutritional deprivation, playing an important function in both the infectivity and virulence to the vertebrate host [182]. During the *T. cruzi* life cycle, epimastigotes are submitted to starvation in the insect rectum, a crucial event for protozoa differentiation. Starved epimastigotes express Atg8.1, but such expression is decreased in metacyclic forms [82, 92]. Reservosomes disappeared during

| Molecules | Organism | References |
|---|---|---|
| Atg4.1, Atg4.2 | *L. major* | [197] |
| Atg5 | *L. major* | [190] |
| Atg5, Atg10, Atg12 | *L. major* | [183] |
| Atg8A, Atg8B, Atg8.2 | *T. brucei* | [183] |
| Atg8.1, Atg8.2 | *T. cruzi* | [92] |
| Atg8, Atg8A, Atg8B, Atg8C | *L. major* | [183] |
| TOR1<br>TOR2 | *T. brucei*<br>*L. major* | [186, 198, 199] |
| TOR3 | *L. major* | [199] |
| Vps34 | *T. cruzi*<br>*T. brucei* | [200, 201] |

**Table 4.** Autophagic molecules described in pathogenic trypanosomatids

differentiation, most likely due to the cysteine proteinase activity, in particular, cruzipain [159, 191, 192].

In *Leishmania* sp., autophagy is essential for metacyclogenesis, with several observed autophagosomes during the process [193, 194]. The deletion of Atg4.2 led to an accumulation of Atg8 lipidated isoforms, compromising the autophagic activity. Subsequently, a reduction in the number of differentiating promastigotes was observed [194]. Interestingly, autophagy also participates in the differentiation of *L. mexicana* metacyclic promastigotes to amastigotes [193]. In the sandfly, the exposure of promastigotes to different stress stimuli, including higher temperature, low pH, and nutritional deprivation, acts as a crucial event for the success of the metacyclogenesis [189, 192]. *L. mexicana* shows that lysosome-like structures, called megasomes, are involved in parasite differentiation, with the activity of two megasomal cysteine peptidases (CPA and CPB) associated with autophagy. The deletion of these proteases strongly impaired its differentiation into amastigotes, leading to an accumulation of autophagosomes containing multi-vesicular tubules (structures related to endocytosis) [180, 193].

A peculiar role for autophagy was observed in *T. brucei*. In a selective pathway, glycosomes are degraded during differentiation from bloodstream to procyclic forms. This organelle is a peroxisome-like structure that is also involved in the glycolytic pathway, and its degradation via autophagy led to important changes in the protozoa bioenergetics [195]. This evidence supported the existence of pexophagy in trypanosomes, an essential event for energy balance during the parasite life cycle. Depending on the environmental conditions (distinct hosts), the sources of energetic substrates vary, as does the ATP demand [180]. Recently, it was also reported that *T. brucei* acidocalcisomes (an acidic compartment that stores ions responsible for polyphosphate metabolism) regulate autophagy by the acidification of this organelle. Moreover, the blockage of acidocalcisome biogenesis also inhibited the autophagic pathway without

the impairment of lysosomal biogenesis or function, suggesting the relevance of acidocalcisomes as an autophagic regulator [196].

Autophagic cell death occurs when the homeostatic balance is broken [40]. To evaluate whether autophagy participates in the cell death process, the use of the PI-3K inhibitors wortmannin and 3-methyladenine (3-MA) before the autophagic stimulus is provided is an interesting experimental approach. Pre-treatment with these inhibitors totally abolished the trypanocidal activity of naphthoimidazoles in *T. cruzi* epimastigotes and trypomastigotes. Although the involvement of components of the Atg8 conjugation system was also demonstrated, the molecular mechanisms of cell death regulation in this parasite deserve further examination [82, 178].

### 3.3. Necrosis

As described for higher eukaryotes, necrosis is poorly studied in protozoa, especially due to its conception as an accidental and uncontrolled process. The most typical necrotic feature is the plasma membrane rupture that leads to the loss of cellular homeostasis and consequent cell lysis as the consequence of a mechanical or chemical stimulus [103]. Necrosis is always the cell death endpoint, culminating in the generation of cellular debris. Thus, independent of the cell death mechanism that is induced, all parasites will lyse in a system without phagocytic cells to clean the microenvironment. In this context, a high percentage of anti-trypanosomatid natural or synthetic drugs present a mechanism of action with a lytic effect [29, 149, 202-205] (Table 3).

Another crucial stress condition that induces trypanosomatid disruption is the activation of the complement pathway. This cascade can be triggered by the binding of lectins to lipophosphoglycans presented on the surface of *Leishmania* sp. promastigotes and of glycosylated molecules in the *T. cruzi* metacyclic form [206-209]. Indeed, pathogenic trypanosomatids show different mechanisms to evade the complement pathway. For example, *T. brucei* expresses a vast number of variant surface glycoproteins (VSG) that change the parasite coat to escape from the host immune system [210]. In relation to programmed necrosis, RIPK-like molecules have not yet been identified in unicellular organisms, and the direct effect of Nec-1 has not been evaluated, suggesting that an orchestrated pathway similar to necroptosis is absent in trypanosomatids.

### 3.4. Others

Curiously, no studies have been reported about non-canonical PCD pathways in trypanosomatids. Pyroptosis and NETosis are processes that are characterized exclusively in mammalian cells, specifically during an inflammatory response. Such pathways involve the death of immune cells to block the progression of any infection by a well-regulated mechanism [106, 110]. The absence of these PCD types in unicellular organisms is not strange. On the other hand, the existence of specific oxidative stress-related cell death types in trypanosomatids would be reasonable. Continuous exposure of these parasites to ROS under distinct environmental conditions during their life cycles indicates the important role of oxidative stress in the

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control of protozoa populations. ROS involvement in trypanosomatid apoptosis-like processes and autophagy has been described in different experimental conditions [130, 155, 156, 211, 212], but ferroptosis has not yet been investigated. Further studies about the effect of erastin as well as the inhibition by ferrostatin 1 should be performed in these parasites. Autoschizis was only observed in cancer cells under very specific conditions, but interestingly, an autoschizic phenotype (high percentages of AV-/PI+ cells) was detected in *T. cruzi* treated with naphthoimidazoles [178]. The AV-/PI+ population is ignored in the majority of the studies, including in pathogenic trypanosomatids [213-215]. A better characterization of this parasite population must be performed to exclude the existence of autoschizis in protozoa.

### 3.5. Cell death and evasion of host immune response

Trypanosomatids presented a highly sophisticated repertoire to evade mammalian immune systems, including the capacity to prevent the cell death pathways of the infected host cells [188]. This efficient strategy allows host PCD modulation by the parasites to establish the infection. Depending on the protozoan species and the host cell type, PCD exacerbation or inhibition fluctuates. For example, the induction of apoptosis in immune cells increases the parasite persistence and survival in immunocompetent hosts [78]. In *T. cruzi* infection, apoptosis of lymphocytes and macrophages is essential for the parasite to escape, promoting inflammation reduction by anti-inflammatory cytokines and also amastigote proliferation [78, 216, 217]. The *Leishmania* strategy is quite different. Promastigotes externalize PS to be recognized by phagocytic cells. The binding of PS to its receptor on the phagocyte surface triggers a signalling cascade that guides TGF-β production and the subsequent anti-inflammatory response. This phenomenon, called apoptotic mimicry, facilitates parasite internalization and increases the success of the infection [218, 219]. Additionally, the intracellular cycle of *Leishmania* sp. also depends on the impairment of host cell apoptosis. This event is necessary to stop or delay the elimination of infected cells. For example, *L. major* uses the infected apoptotic granulocytes as "Trojan horses" to invade macrophages, the definitive host cells, avoiding the direct activation of phagocytes via the interaction between host receptors and protozoa [220].

Host autophagy also represents a valuable mechanism for both innate and adaptive responses to stop the infection. Its blockage is a crucial tactic for pathogenic trypanosomatids to evade host defences. Autophagy uses a process to eliminate pathogens, called xenophagy, directing microorganisms to be digested in lysosomes. This strategy is usually employed by protozoa living inside parasitophorous vacuoles to use the autophagic machinery to provide nutrients [82]. However, protozoa, such as *Leishmania* sp., change the autophagosomal pH and impair vesicular traffic, compromising the fusion to lysosomes. *L. amazonensis* amastigotes proliferate in starvation or even after treatment with rapamycin, but the proliferation is inhibited by incubation with the autophagic inhibitors wortmannin or 3-methyladenine [221]. The importance of autophagy for the *Leishmania* infection was corroborated by the observation that this pathway is exacerbated in *L. amazonensis*-infected mice and in a natural *L. donovani* infection in humans [222, 223]. Similar data were observed in the *in vitro T. cruzi* infection, suggesting that the autophagic pathway favours the parasite during its interaction with the host cell [224,

225]. However, the role of host autophagy in this trypanosomatid is still controversial due to the autophagic participation in the control of *T. cruzi* infection [226-228]. Furthermore, differences among strains and host cells must be considered to clarify whether host autophagy kills *T. cruzi* or provides nutrients for its survival.

## 4. Concluding remarks

In spite of the variety of studies about cell death in protozoans, including trypanosomatids, and the evidence of PCD, the detailed aspects of the molecular mechanisms and regulation remain unclear. The absence of key molecules together with the lack of an obvious role for this process in unicellular organisms makes the existence of PCD in these cells a debatable point, and the term "apoptosis-like" is more convenient [130, 172, 229]. In this context, the lack of a strong correlation between the proteolytic properties of caspases and their role in PCD should be highlighted. Currently, there is no description of the participation of trypanosomatid metacaspases in cell death processes, but these proteases have been postulated to function in proliferation and differentiation, which are important events for parasite survival [145, 148, 149, 153, 230]. In the post-genomic era, a rigorous search should be performed in proteomic databases of pathogenic trypanosomatids to correct misannotations in cell death proteins, validating the real role of these molecules for PCD processes.

Nevertheless, PCD was conserved during evolution, suggesting its essential function for the survival and maintenance of these species. However, it has been proposed that these pathways appeared in the phylogenetic tree in the multicellular organism branches, suggesting that the death molecular mechanisms identified in unicellular parasites came from a divergent evolutionary event [48]. This idea is supported by the replacement or complete absence of some PCD molecules, justifying the differences observed in protozoa mechanisms [79]. In addition to being an interesting evolutionary model for PCD, its physiological relevance for protozoa is still the most attractive question.

An altruistic hypothesis has been raised for unicellular organisms, especially for pathogenic trypanosomatids [130]. It was associated with the control of parasite populations, including protozoa density regulation, clonal selection and immune host system evasion, events related to the success of the infection [7, 76, 82, 136, 231]. Trypanosomatid cell death limits parasite colonization in insects in response to scarce resources of nutrients, avoiding invertebrate death [118, 130, 134]. On the other hand, PCD of *T. cruzi* or *L. amazonensis* insect forms under mammalian temperatures could evade host immune response derived from parasite lysis, facilitating the infection [76, 119, 135].

Autophagic cell death has been proposed as a PCD pathway, suggesting an active role of autophagy in death processes, but the precise mechanisms of regulation are not yet clear [174, 178, 232]. The majority of the autophagic studies were performed in yeast and mammal models. However, little is known about protozoan pathways. Autophagy is a regulated process that is directly involved in the preservation of cellular homeostasis and survival. Several hypotheses have been raised about the participation of this pathway in cell death in dying cells. The selective autophagic degradation of essential cellular factors, such as cell death regulators,

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triggers death events, including caspase activation [232, 233]. Another hypothesis suggested that autophagy is not a specific and regulated cell death process but is a consequence of extensive injury. Once such an injury compromises cellular physiology, the damaged structure needs to be degraded for cell survival. This hypothesis also explains the presence of similar phenotypes in parasites after treatment with different compounds with distinct mechanisms of action. Such autophagic phenotypes, detected independent of the stimuli, reinforced this pathway as a desperate attempt of the cells to stay alive [168, 212, 232]. The determination of the connection between the autophagic cell death of pathogens, such as trypanosomatids, could have crucial implications for human health, but further mechanistic studies should be addressed in this field.

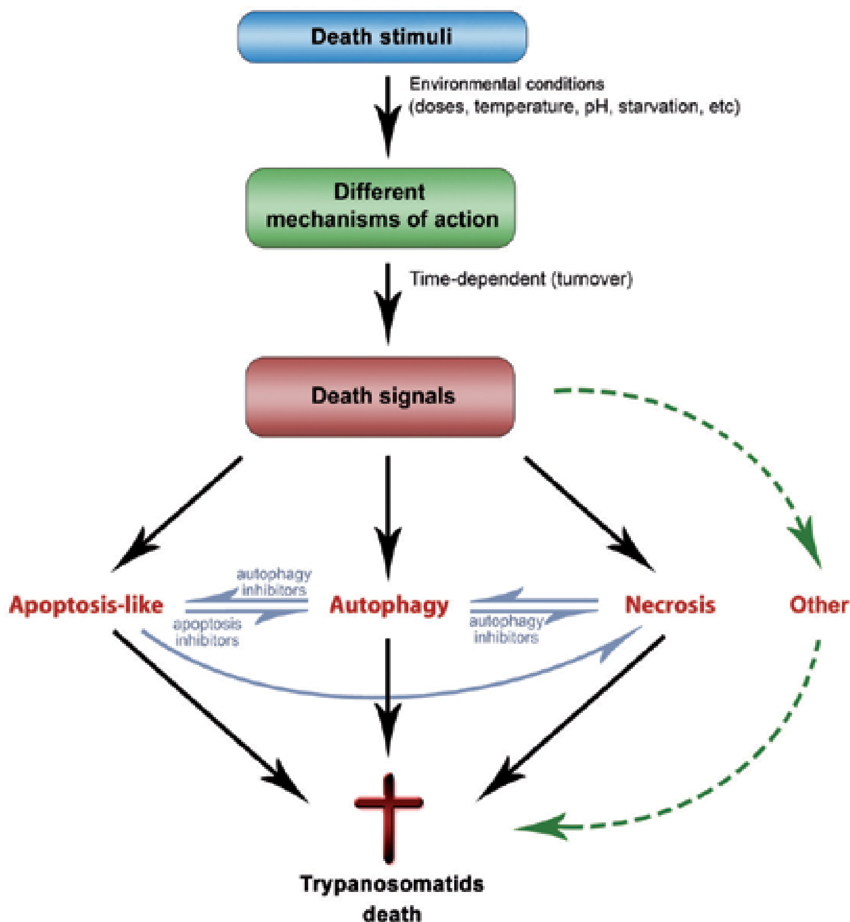


**Figure 3.** Different pathways of trypanosomatid death. The death stimulus triggers specific mechanisms of action depending on the environmental conditions, time of treatment and dose. Death signals lead to distinct well-known phenotypes from each pathway. Cross-talk could also be observed between apoptosis-like processes, autophagy and necrosis, culminating in protozoa death. The existence of an alternative unknown process cannot be discarded (dashed arrow).

The existence of cross-talk among different cell death pathways, especially autophagy and apoptosis, has been proposed (Figure 3) [93, 234]. In unicellular parasites, different cell death types have been described to be induced by physical and/or chemical stress conditions (drugs, heat shock, and nutritional deprivation, among others), resulting in a non-classical cell death phenotype. The total absence of commercial typical PCD markers, such as antibodies and enzyme activity kits, for protozoa and of key autophagic and apoptotic-like molecules reinforce the hypothesis of an interplay of distinct death mechanisms, suggesting their convergence, leading to necrosis. Likewise, the possibility of the occurrence of other PCD forms cannot be excluded [74, 78, 168, 178]. A better molecular characterization of cell death in pathogenic trypanosomatids is essential for advances in novel alternatives for therapeutic intervention.

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Chapter 12

# Apoptosis and Infections

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Additional information is available at the end of the chapter

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**Abstract**

Apoptosis is a process that plays a critical role in the elimination of infected cells. Infectious diseases modulate apoptosis, and this contributes to disease pathogenesis. Apoptosis is initiated by various kinds of stimuli, including infections, radiation, etc. Increased apoptosis may assist the dissemination of intracellular pathogens or induce immunosuppression. However, apoptosis may also help eradicate pathogens from the host in many cases. Consequently, several viruses, bacteria, and parasites have evolved mechanisms to inhibit host cell by apoptosis as a strategy that may support intracellular survival and persistence of the pathogen. Bacteria are recognized by cellular receptors and elicit a multitude of signal transduction events that alter the cell's response toward apoptotic stimuli. The result of pathogenic bacteria entering into mammalian cells evokes variety of responses, including internalization or phagocytosis of the bacteria, release of cytokines, secretion of defensins, production of oxygen radicals and the triggering of apoptosis. Bacteria can trigger apoptosis through a large variety of mechanisms that include the secretion of protein synthesis inhibitors and pore forming proteins. They can also activate apoptotic proteins such as caspases, inactivate antiapoptotic proteins, or lead to upregulation of the endogenous receptor/ligand system. However, new research has shown that many bacterial pathogens can in fact prevent apoptosis during infection. As in bacteria, many viral genomes encode proteins that repress apoptosis to escape from immune attack by the host or viruses promote apoptotic death of the host cells. Virus-host interactions may determine viral persistence, extent and severity of inflammation, and pathology associated with infectious disease. The elucidation of the signaling pathways, the cellular receptors, and/or the microbial factors involved in the induction or reduction of apoptosis could reveal new therapeutic targets for blocking microbial-induced apoptosis. This chapter will summarize the most recent research on microorganisms' apoptotic and antiapoptotic strategies and the mechanisms relating to disease.

**Keywords:** apoptosis, infection, bacteria, viruses, parasitis

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# 1. Introduction

## 1.1. Apoptosis regulators

Programmed cell death or apoptosis is an intrinsic death program that occurs in various physiological and pathological situations. Apoptosis is also a physiological process that is critical for tissue homeostasis. It is essential for the regulation of immune responses. The main regulators of apoptosis are caspases, Bcl-2 family, p53, tumor necrosis factor (TNF) family, and/or inhibitors of apoptosis proteins (IAPs).

# 2. Caspases

Caspases are a family of proteins that are one of the main effectors of apoptosis. Their activation is a hallmark of apoptosis. Caspases are synthesized as inactive zymogens, the so-called procaspases. Upon maturation, the procaspases are proteolytically processed between the large and small subunit resulting in a small and a large subunit.

Based on their function, the caspases can be classified into three groups: (1) inflammatory caspases—this group includes caspases 1, 4, 5, 11, 12, 13, and 14, which are involved in inflammation instead of apoptosis; (2) apoptotic initiator caspases that possess long prodomains containing either a death effector domain (DED) (caspases 8 and 10) or a caspase activation and recruitment domain (CARD) (caspases 2 and 9), which mediate the interaction with upstream adaptor molecules; and (3) apoptotic effector caspases. This executioner class (caspases 3, 6, 7) is characterized by the presence of a short prodomain [1]. Apoptotic signals trigger the oligomerization of death adaptor proteins, while death adaptor oligomers in turn induce the aggregation of procaspases. It was previously believed that the initiator caspases are autoproteolytically activated when brought into close proximity of each other. This is called the "induced proximity" model. Effector procaspases are normally cleaved and activated by active initiator caspases. They then cleave various death substrates to induce cell death [2].

# 3. IAPs

The IAPs represent a family of evolutionarily conserved apoptosis suppressors. Although IAP family proteins may possess other functions, several of them have been shown to bind and potently inhibit activated caspases. Among the caspases inhibited by human IAP family members, XIAP, cIAP1, and cIAP2 are the effector caspases 3 and 7 as well as the initiator caspase 9 [3]. IAP expression can be upregulated in response to survival signals such as those coming from growth factor receptors, e.g., by the activation of the transcription factor. Nuclear factor-kappa B (NF-κB), however, provides a means to suppress apoptosis signaling. IAP inhibitor SMAC/Diablo was recently described as those that bind multiple IAP family members and those that allow caspases to induce apoptosis [4].

## 4. Bcl-2 family

The Bcl-2 family consists of both antiapoptotic and proapoptotic proteins that share sequence homology within conserved regions known as Bcl-2 homology (BH) domains. All antiapoptotic members such as Bcl-2 and Bcl-$_{XL}$ and a subset of proapoptotic family members such as Bax and Bak are multidomain proteins sharing sequence homology within three to four BH domains [5].

## 5. p53

One of the most important p53 functions is its ability to activate apoptosis. The disruption of this process can promote tumor progression and chemoresistance. p53 tumor suppressor protein blocks cell cycle progression allowing time to repair the damage or induces apoptosis largely through the upregulation of the Bcl-2 family BH3-only protein Puma (p53 upregulated modulator of apoptosis). Many apoptosis-related genes that are transcriptionally regulated by p53 have been identified. p53-dependent apoptosis is frequently the one induced following DNA damage caused by irradiation, UV or viral infections. p53-independent pathways are usually those resulting from growth factor deprivation. The activation of p53 by DNA damage induces either cell cycle arrest or apoptosis. p53 mediates apoptosis through a linear pathway that involves Bax transactivation, Bax translocation from the cytosol to membranes, cytochrome *c* release from mitochondria, and caspase 9 activation followed by the activation of caspases 3, 6, and 7 [6,7].

### 5.1. Apoptosis signaling pathways

Apoptosis can be induced in response to various signals from inside and outside the cell. Apoptosis process involves two pathways: (1) by the release of cytochrome *c* from mitochondria—intrinsic pathway and/or (2) by the activation of cell-surface death receptor—extrinsic pathway [8].

### 5.2. Intrinsic pathway

Mitochondria is a central regulator of intrinsic apoptotic pathways. Intrinsic apoptotic pathways are initiated inside cells. Numerous cytotoxic stimuli and proapoptotic signal-transducing molecules converge on mitochondria to induce outer mitochondrial membrane permeabilization. Mitochondria are known as an important intracellular organelle for producing energy. Mitochondria also play a key role in the modulation of $Ca^{2+}$ homeostasis and oxidative stress. The dysfunction of mitochondria induced by DNA damage or other genotoxic factors leads to an irreversible event, apoptotic cell death. The intrinsic apoptotic pathway is also called "mitochondrial pathway." A pivotal event in the mitochondrial pathway is mitochondrial outer membrane permeabilization (MOMP). MOMP is mainly mediated and controlled by Bcl-2 family members [9]. Many proteins of the Bcl-2 family either with antiapoptotic (e.g., Bcl-2, $Bcl_{XL}$, or Mac1) or proapoptotic (Bax, Bak, or Bik) functions reside in the

outer membrane of the mitochondria. In healthy cells, a small proportion of Bak molecules are bound to voltage-dependent anionic channel (VDAC; part of the [permeability transition] [PT]). The antiapoptotic molecules Bcl-2 and $Bcl_{XL}$ prevent the translocation of cytochrome *c* from the mitochondria, while the induced expression or enforced dimerization of Bax results in dysfunction leading to cytochrome *c* release [10].

Specific stimuli such as oxidants, calcium overload, or ceramide cause a decrease in mitochondrial inner transmembrane potential ($\Delta\Psi_m$) and result in the release of cytochrome *c* from the mitrochondrion. Active Bax/Bak causes the release of cytochrome *c*, which then binds to APAF-1 and causes its oligomerization. The release of cytochrome *c* from the mitochondrial intermembrane space to the cytosol contributes to the formation of the apoptosome that consists of cytochrome *c*, APAF-1, and dATP. Caspase 9 is recruited into the complex and activated in this process. The apoptosome activates caspase 9, which is another initiator caspase. Active caspase 9 cleaves and thereby activates effector caspases (most notably caspase 3), and active effector caspases cause the morphological signs of apoptosis by cleavage of other effector proteins [11]. During apoptosis, cells undergo several morphological and biochemical changes. Due to endonuclease activation, the chromatin is cleaved into oligonucleosomal fragments. Recently, it was shown that structural changes in the plasma membrane of the apoptotic cell are functional in signaling the process of cell death to the environment [12]. Active proteases, including caspases, calpains, cathepsins, and/or serine proteases, can promote the activation of DNases in different ways. Effector caspases cleave and inactivate DNA repair enzyme poly-ADP-ribose polymerase (PARP). Regulators of the cell cycle such as retinoblastoma protein and structural proteins of the nucleus and cytoskeleton such as lamins, growth arrest-specific protein 2, gelsolin, fodrin, and survival proteins such as protein kinase C-δ (PKC-δ) cause cell death [13].

Caspase 3 is responsible for degradation of the nuclear protein PARP, which is involved in DNA repair. Apoptosis inducing factor (AIF) is a proapoptotic factor in mitochondria. It triggers chromatin condensation and DNA degradation in a cell in order to induce apoptosis [14].

### 5.3. Extrinsic pathway

The extrinsic pathway is activated by ligand-bound death receptors, mainly including (a) TNF-TNFR1, (b) FasL-Fas, and (c) TNF-related apoptosis-inducing ligand (TRAIL) DR4 or DR5. Death receptors belong to the tumor necrosis factor receptor gene (TNFR) superfamily and can generally have several functions that include initiating apoptosis. The TNFR superfamily is characterized by the presence of cysteine-rich domains that mediate binding between ligands and these type I transmembrane domain receptors. Among them, the death receptors, including TNF-R1, Fas (or CD95), and the TRAIL receptors DR4 and DR5, are best characterized for induction of apoptosis [15].

#### *5.3.1. TNF pathway*

TNF is a multifunctional proinflammatory cytokine mainly produced by macrophages. There are two major TNF receptors, TNF-R1 and TNF-R2. TNF-R1 is ubiquitously expressed in most

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tissues and is the major mediator of TNF signaling, whereas TNF-R2 is mainly found in the immune system and only can be fully activated by membrane bound TNF [16].

TNF-induced activation of NF-κB, JNK, and apoptosis has been intensively studied. NF-κB is a transcription factor that can be induced by a variety of signals. Inhibitor of KB (I-KB) binds to NF-κB and inactivates it by localizing NF-κB to the cytosol, where it is unable to regulate transcription. The phosphorylation of I-KB targets it for ubiquitination and degradation. In the absence of I-KB, NF-κB's nuclear localization signal is exposed, and NF-κB localizes to the nucleus where it is able to induce transcription. The role of NF-κB was originally described as a factor associated with apoptosis. This process is triggered by the phosphorylation of I-KB by the I-KB kinase (IKK) complex. Different activation pathways of NF-κB may cause the expression of proteins that promote apoptosis (e.g., Fas, c-myc, p53, TNF, DR, and caspase 11) or inhibit apoptosis (e.g., IAP proteins, Bcl-2-like proteins) [17]. It was subsequently shown that the inhibition of NF-κB activation potentiates apoptosis [18]. In addition, the inhibition of I-KB expression by oligonucleotides led to cell transformation. Consistent with this role, NF-κB has been shown to induce transcription of antiapoptotic proteins. Akt is reported to phosphorylate and activate IKK. The activation of IKK causes phosphorylation and degradation of I-KB, which leads to the localization of NF-κB to the nucleus where it can induce transcription of antiapoptotic genes. Thus, Akt can inhibit apoptosis by activating NF-κB [19].

TNF also induces the activation of the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) pathway. Upon activation, JNK kinases translocate into the nucleus and enhance the transcriptional activity of transcription factors, for example, c-Jun and activating transcription factor-2 by the phosphorylation of their amino-terminal activation domains. c-Jun belongs to a group of basic region-leucine zipper proteins that dimerize to form transcription factors commonly designated as activator protein 1 (AP-1). The AP-1 proteins have an important role in a variety of cellular processes, including proliferation, differentiation, and induction as well as prevention of apoptosis [20].

#### *5.3.2. FAS pathway*

Fas is involved in the cytotoxic T lymphocyte (CTL)-mediated killing of cells (e.g., CTL-mediated killing of virus-infected cells), destruction of inflammatory and immune cells in immune-privileged sites, and deletion of self-reacting B cells and activated T cells at the end of an immune response [21]. Fas binding to Fas ligand results in intracellular clustering of death domains (DD) followed by its internalization into an endosomal pathway. This allows an adaptor protein called Fas-associated death domain (FADD) to associate with the receptor through an interaction between homologous death domains on both molecules. FADD also contains a DED that allows binding of procaspase 8 to the CD95-FADD complex. Procaspase 8 (also known as FLICE) associates with FADD through its own death effector domain. Caspase 8, the main initiator caspase in CD95 signaling, is expressed as two isoforms, caspases 8/a and 8/b, which are both recruited to the activated CD95 receptor. FasL-induced clustering of Fas, FADD, and caspase 8 within the death-inducing signaling complex leads to autoproteolytic processing of caspase 8 by induced proximity and dimerization followed by the release of the processed active proteases [22]. Cells can be divided into two types according to their require-

ment for mitochondrial pathway in FAS-induced apoptosis. In type I cells, processed caspase 8 is sufficient to directly activate other members of the caspase family. In type II cells, the efficient activation of effector caspases by Fas depends on an amplification loop that relies on caspase-8-mediated cleavage of Bid and subsequent release of mitochondrial proapoptotic factors such as SMAC/Diablo or cytochrome *c* to drive the formation of the caspase-9-activating apoptosome. Active caspase 9 activates the executioner caspase 3, which in turn activates caspase 8 [23].

*5.3.3. TRAIL pathway*

Like Fas, TRAIL may also be involved in the immune response and in tumor surveillance. Five distinct TRAIL receptors have been identified: death receptor 4 (TRAIL-R1), 125 KILLER/DR5 (TRAIL-R2, TRICK2), DcR1 (TRID, TRAIL-R3), DcR2 (TRUNDD OR TRAIL-R4), and osteoprotegerin. The apoptotic signaling induced by TRAIL is similar to that induced by FAS. Binding of TRAIL to its receptors DR4 or DR5 triggers the formation of a death-inducing signaling complex by recruiting FADD and/or caspases 8 and/or 10. TRAIL-induced apoptosis also involves the mitochondrial pathway, like Fas-induced apoptosis, in type II cells [24,25].

## 6. Apoptosis and bacterial infections

During a microbial infection, organism faces the challenge of recognizing and combating the invading organism. The entrance of bacterial pathogens into human organism initiates the innate immune response characterized by the recruitment of leukocytes to sites of infection [26]. After phagocytosis by human macrophages, microorganisms are destroyed by reactive oxygen species (ROS) microbicidal products contained within granules [27]. Otherwise, bacterial pathogens that cause apoptosis target immune cells such as macrophages and neutrophils [28]. Bacteria can trigger apoptosis by different mechanisms, including the secretion of protein synthesis inhibitors, pore forming proteins, molecules activating the endogenous death machinery in the infected cell, or lipopolysaccharides and other superantigens [29]. These molecules might be either proapoptotic and activated by the bacteria or antiapoptotic and inhibited upon infection to trigger apoptotic death of the infected cell [30] (Table 1).

| **Bacteria** | **Apoptosis** | **Proposed or demonstrated mechanism** | **Cell type** |
|---|---|---|---|
| *Pseudomonas aeruginosa* | Induction | Fas/Fas ligand system | Endothelial cells |
| | | Cytochrome *c* release | Epithelial cells |
| *Neisseria gonorrhoeae* | Induction | Increases mitochondrial permeability | Epithelial cells |
| | Inhibition | Increases antiapoptotic genes | PMLs |
| | | | Epithelial cells |
| *Neisseria meningitidis* | Inhibition | Prevents cytochrome *c* release | HeLa cells |

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| *Shigella flexneri* | Induction | Caspase 1 activation | Macrophages |
|---|---|---|---|
| | Inhibition | Prevents cytochrome *c* release | Epithelial cells |
| Shiga toxins | Induction | Extrinsic and intrinsic pathways | Epithelial, endothelial, neurons |
| | | | myeloid and lymphoid cells |
| *Salmonella typhimurium* | Induction | Caspase 1 activation | Macrophages |
| | Inhibition | PI3K-Akt/PKB-pathway | Epithelial cells |
| *Listeria monocytogenes* | Induction | Cytochrome c release | Hepatocytes, lymphocytes |
| | | | dendritic cells |
| *Chlamydia trachomatis* | Inhibition | Prevents cytochrome *c* release | Epithelial cells, macrophages |
| *Chlamydia pneumoniae* | Inhibition | Bcl-2 | HeLa cells |
| | | Raf/MEK/ERK survival pathway | |
| *Yersinia pseudotuberculosis* | Induction | Inhibits ERK and the NFKB | Macrophages, dendritic cells |
| *Yersinia enterocolitica* | | TLR4 | |
| *Yersinia pseudotuberculosis* | Inhibition | Inhibits ROS production | PMLs |
| *Yersinia enterocolitica* | | | |
| *Yersinia pestis* | Induction | Caspase 1 activation | Macrophages |
| | | Inhibits ERK and the NFKB | |
| *Legionella pneumophila* | Induction | Caspase 3 | Macrophages, epithelial cells |
| | Inhibition | Up regulating of anti-apoptotic genes | Monocyte, U937,A549 cells |
| | | NF-κB pathway | |
| *Escherichia coli* K1 | Induction | Extrinsic and intrinsic pathways | Epithelial cells |
| | Inhibition | Expression $Bcl_{XL}$ | Macrophages |
| *Rickettsia rickettsii* | Inhibition | Activation of cell survival pathways | Endothelial cells |
| | Induction | DNA fragmentation | Neurons |
| *Mycobacterium tuberculosis* | Induction | TNF pathway | Macrophages |
| | | Intrinsic pathway | |
| | Inhibition | Activation of the NF-κB | Epithelial cells |
| | | Expression of Bcl-2 | |
| **Viruses** | **Apoptosis** | **Proposed or demonstrated mechanism** | **Cell type** |
| Hepatitis C virus | Induction | Extrinsic pathway | Cytotoxic T lymphocytes, macrophages |

| | | | |
|---|---|---|---|
| | Inhibition | Inhibits both Fas pathway and intrinsic pathway | Hepatocytes |
| HIV-1 gp120 protein | Induction | Fas pathway | CD4+ T cells |
| HIV-1 proteins Env | Induction | p53-dependent genes Puma and Bax | |
| HIV Nef | Induction | Extrinsic pathway | |
| Rabies virus | Induction | Expression of Bax and caspase 1 | Neuroblastoma cell |
| | Induction | Caspase gene Nedd-2 | Neurons |
| | | Activation of caspase 8 | |
| | | Upregulation of AIF | |
| Epstein-Barr virus | Inhibition | Bcl-2 proteins, BHRF1, and BALF1 | B cells |
| | | NF-κB pathway | lymphoblastoid cell lines |
| | | Upregulation of antiapoptotic genes | |
| Baculovirus | Inhibition | Antiapoptotic genes (p35 and IAP) | Insect cells |
| HPV E2 protein | Induction | p53 pathway | HeLa cells |
| HPV E6 protein | Induction | Degradation p53 pathway | Epithelial cells |
| | Induction | Extrinsic pathway | Cervical carcinoma cells |
| | Inhibition | Caspase inactivation | fibroblasts, osteosarcoma cells |
| HPV E7 protein | Induction | Retinoblastoma gene | Lens |
| Adenoviral proteins | | | |
| E1A | Induction | p53 pathway | REF52 cells |
| E4orf4 | Induction | p53 pathway, caspase activation | H1299, 293 cells |
| E4orf6 | Induction | PARP-induced cell death | U251 cells |
| E1B-19K | Inhibition | Inhibits extrinsic pathway | HeLa cells ,A549 lung carcinoma cells |
| E1B-55K | Inhibition | Inactivates p53 | 293 cells |
| E3-6.7 | Inhibition | Blocks TRAIL-induced apoptosis | Cytotoxic T cells |
| E3 RID | Inhibition | Inhibits E1A or TNF-induced apoptosis | HT29.14S cells |
| | | Decreased presentation of Fas on the cell surface | Jurkat, CEM, and HuT78 cell lines |
| E3-14.7K | Inhibition | Inhibits TNF and TRAIL-induced apoptosis | Fibroblast C3HA cell lines |
| | Inhibition | inhibits MHC class I transport to the cell surface | T lymphoma cell line |
| E4 orf 6 | Inhibition | Blocks p53 | H1299 cells |
| Human cytomegalovirus | | | |

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| UL36 protein | Inhibition | Inhibits Fas and caspase-mediated apoptosis | MRC-5 fibroblasts, HeLa cells |
|---|---|---|---|
| | Inhibition | Inhibits TNF-induced apoptosis | HeLa cells |
| **Parasites** | **Apoptosis** | **Proposed or demonstrated mechanism** | **Cell type** |
| *Toxoplasma gondii* | Inhibition | Increased antiapoptotic Bcl-2 family | Fibroblasts |
| | | Inhibition of the cytochrome *c* release | |
| | | Upregulation of IAPs | |
| | | Activation of NF-κB | |
| | | Degradation of PARP | |
| | Induction | IFN-γ | T cell |
| *Plasmodium falciparum* | | Increased Ca level | Erythrocytes |
| | | Caspase 8 and caspase 9 activation | Endothelial cells |
| *Trypanosoma brucei* | Induction | Intrinsic pathway | Neuronal cell |
| *Trypanosoma cruzi* | Inhibition | PI3-K/PKB pathway | Schwann cells |
| *Leishmania donovani* | Induction | Intrinsic pathway | Neutrophils |

**Table 1.** Microorganisms that induce and/or inhibit host cell apoptosis

Apoptosis is induced by both intrinsic and extrinsic pathways. Due to their key role in cell survival, mitochondria represent attractive targets for pathogens. Several pathogens, including both viruses and bacteria, have been shown to target mitochondria in order to interfere with the host cell apoptotic machinery. For example, bacterial pore-forming toxins such as the neisserial porin PorB, which causes rapid $Ca^{+2}$ influx into target cells and induces apoptosis, exhibit striking structural and functional homology with the mitochondrial anion channels that mediate mitochondrial permeability transition and apoptosis [31]. Apoptosis, which is executed by caspase activity, can be induced either by the ligation of death receptors or by the release of another proapoptotic factor cytochrome *c* from mitochondria. Apoptotic stimuli (either death ligands, binding to death receptors, or any of the multitude of agents that induce apoptosis) are received by some cellular receptor. In the case of death receptors, this directly causes caspase activity [32].

New studies have shown that many bacterial pathogens can prevent apoptosis during infection. Bacteria inhibits apoptosis by the use of multiple mechanisms: the protection of the mitochondria and prevention of cytochrome *c* release (i.e., *Chylamidia* sp. and/or *Neisseria* sp.), the activation of cell survival pathways (i.e., *Salmonella* sp. and/or *Rickettsia* sp.), the inhibition of caspases, and the activation of phosphoinositide 3-kinase (PI3K)-Akt/protein kinase B (PKB) pathway and interaction with cellular caspases (i.e., *Shigella* sp. and/or *Legionella* sp.) [33]. The PI3K/Akt pathway is also a strong activator of cyclin D1, a critical player in cell cycle progression. Cyclin D1 protein levels are also regulated by glycogen synthase kinase 3 (GSK-3). GSK-3 is the primary kinase that phosphorylates cyclin D1 at this residue. Rapid cyclin D1 degrada-

tion induced by GSK-3 is inhibited by the activation of PI3K/Akt pathway because Akt directly phosphorylates and inactivates GSK-3 [34]. c-Fos is a transcriptional regulator that can elevate the expression of many proliferatory genes. PI3K/Akt pathway executes some of its antiapoptotic effects through transcription factors such as Elk-1 and c-Fos. The activation of the Raf/MEK/ERK pathway is associated with the increased expression of antiapoptotic proteins. The Raf/MEK/ERK cascade can also activate the PI3K/Akt pathway. Raf has been shown to directly phosphorylate Bad and Bcl-2 to exert antiapoptotic effects. Depending on cell type as well as apoptotic stimulus, Raf can inhibit or promote apoptosis [35]. Toll-like receptors (TLR) have been described to have both apoptosis-inducing and inhibiting capacity. Early reports have linked the activation of TLR2 to the induction of apoptosis through the adapter MyD88 [36]. At the same time, TLR-signaling has clearly an antiapoptotic activity via NF-κB and the PI3Kpathways. NF-κB induces antiapoptotic gene expression [37].

### 6.1. *Pseudomonas*

*Pseudomonas aeruginosa* can cause disease in animals, including humans. The symptoms of such infections are generalized inflammation and sepsis. If such colonizations occur in critical body organs such as the lungs, the urinary tract and kidneys, the results can be fatal [38]. *P. aeruginosa* kills mammalian cells by an activation of the endogenous CD95 (Fas)/CD95 ligand (Fas-ligand) system. An upregulation of cell surface CD95 and CD95 ligand resulting in the activation of this death receptor has been recently shown to be pivotal for the induction of apoptosis by several *P. aeruginosa* strains. The upregulation of CD95 and the CD95 ligand on cells infected with *P. aeruginosa* depends on the function of the type III secretion system (T3SS). The ligation of the receptor stimulates caspases, mitochondrial changes, and finally execution of apoptosis *in vitro* and *in vivo* [30]. The binding of CD95 by the CD95 ligand upon upregulation induces the activation of caspases and JNK. In addition, reactive oxygen intermediates in the induction of *P. aeruginosa* triggered death [39].

### 6.2. *Neisseria*

*Neisseria gonorrhoeae* is the etiological agent of the sexually transmitted disease gonorrhea. It penetrates the mucosa, enters phagocytes and epithelial cells, and causes a massive inflammatory response in the subepithelial tissue [40]. Several factors play a role in infection, for example, the pili, which mediates primary adherence; the Opa proteins, which mediate adhesion and invasion; and the PorB porin [41,42]. There is conflicting information regarding the effects of neisserial porins on apoptosis. These discrepancies may be due to the specific responses of different cell types, culture conditions, and bacterial strains. *N. gonorrhoeae* porin PorB1B interacts with HeLa cell mitochondria and induces calcium eflux and apoptosis [43]. Massari *et al*. [44] showed that Neisserial PorB is translocated to the mitochondria of HeLa cells infected with *Neisseria meningitidis* and prevent apoptosis by the inhibition of cytochrome *c* release. Massari *et al.* [44] speculated that differences in cell types or porin purification explain the discrepancies between the results. *N. gonorrhoeae* also increases the transcription of host antiapoptotic genes. These genes include bfl-1, cox-2 and c-IAP-2, each coding for a product that acts to inhibit apoptosis.

Bfl-1 is a member of the Bcl-2 family of apoptotic regulators and has been characterized to have a protective effect on host cells when overexpressed. Anti-apoptotic Bcl-2/Bcl-$_{XL}$ interact with the mitochondrial porin VDAC, thus blocking the opening of the PT and/or mitochondrial membrane depolarization and inhibiting cytochrome *c* release [45]. *N. gonorrhoeae* can inhibit apoptosis induced by the intrinsic and extrinsic apoptosis inducers staurosporine (STS) and TRAIL in HL-60 cells and primary polymorphonuclear leukocytes (PMLs) [46]. In addition Follows *et al.* [47] showed that *N. gonorrhoeae* infection in human endocervical epithelial cells induced NF-κB activation and resulted in the increased gene expression of the NF-κB-regulated antiapoptotic genes Bfl-1 and/or cIAP-2.

### 6.3. *Shigellas*

The genus *Shigella* consists of four pathogenic "species": *S. dysenteriae*, *S. flexneri*, *S. sonnei*, and *S. boydii. S. flexneri* causes dysentery (shigellosis) by invading the human colonic mucosa. It directly activates proapoptotic signaling pathways to initiate apoptosis in macrophages. It crosses epithelium and goes to lamina propria of intestinal villi. The three proposed effectors of *S. flexneri* internalization are invasion plasmid antigens (Ipa) IpaB, IpaC, and IpaD, all of which are encoded on the pathogen's 230-kb virulence plasmid. These effectors cause caspase 1 activation. Activated caspase 1 then cleaves and activates prointerleukin (proIL)-1β and proIL-18, which are proinflammatory cytokines involved in host inflammatory [48,49]. The secretion of Ipa proteins is dependent on T3SS, which is encoded by 20 genes in the mxi-spa locus of the virulence plasmid. Additional T3SS effector proteins are secreted through the T3 needle when the bacteria are inside the cytoplasm of the host cell [50]. *S. flexneri* kills more of macrophages and promotes the spreading of the bacteria because of the release of interleukin 1β (IL-1β). IL-1β recruits PMLs to the infection sites. The PMLs cross the intestinal epithelium altering the integrity of this epithelial barrier. This promotes massive secondary invasion of the bacteria and acute inflammation [51]. *S. flexneri* infects enterocytes from the basolateral side. The ultrastructural morphology of infected macrophages includes condensation of chromatin at the nuclear boundary blebbing at the cell surface, dilation of the endoplasmic reticulum, and cytoplasmic vacuolization. This is identical to the morphology of cells undergoing apoptosis [52]. *Shigella* does not induce apoptosis in epithelial cells because these intestinal cells are the primary sites for intracellular bacterial proliferation during shigellosis. It was shown that in the presence of STS, *S. flexneri* inhibits apoptosis by preventing the activation of caspase 3. This happens because of both cytochrome *c* release from the mitochondria and caspase 9 activation [53]. Faherty *et al.* [54] suggested that *Shigella* is protected from apoptosis in epithelial cells by various mechanisms. The bacteria prevent cytochrome *c* release from the mitochondria through the upregulation of Bcl-2 proteins. Second, the extrinsic pathway of apoptosis is inhibited from *in vivo* stimuli such as TNF and FasL. Third, infection leads to the induced expression of JNK and NF-κB, which has many prosurvival effects, including the increased expression of the IAPs (BIRC4, BIRC1, BIRC5, and BIRC7), the Bcl-2 family, and the caspase 8 inhibitors. Finally, the bacteria prevent caspase 3 activation to provide downstream protection in the presence of strong apoptosis inducers. Through the use of T3SS effector proteins, the bacteria could directly generate mitochondrial protection, extrinsic pathway inhibition, and caspase 3 inhibition [54].

Shiga toxins comprise a family of structurally and functionally related protein toxins expressed by *S. dysenteriae* serotype 1 and multiple serotypes of *Escherichia coli.* Shiga toxins cause bloody diarrheal diseases, which may progress to life-threatening extraintestinal complications. The kidneys and central nervous system are the organs most frequently involved [55]. Shiga toxins induce apoptosis in human epithelial, endothelial, myeloid, and lymphoid cells *in vitro* and appear to induce apoptosis in rabbit neurons *in vivo*. Apoptosis induction involves the activation of both extrinsic (caspases 8, 6, and 3 activation) and intrinsic (Bid generation, cytochrome *c* release, and/or caspase 9 activation) pathways. Alterations in the balanced expression of pro- and antiapoptotic Bcl-2 family members also contribute to apoptosis induction [56].

### 6.4. *Salmonellas*

*Salmonella* cause an acute localized inflammation in the intestine. *Salmonella enterica* serovar *Typhimurium*, one of the most common food-borne pathogens, causes self-limiting gastroenteritis in humans and a similar diarrheal disease in calves and pigs. *Salmonella typhimurium* can access systemic tissue (mainly spleen and liver) via the lymphatic system and the Peyer's patches. *Salmonella* uses specific virulence factors to invade other cell types such as T3SS. *S. typhimurium* directly activate apoptosis in macrophages [57]. *Salmonella* invasion protein (Sip)B activates ICE (IL-1β converting enzyme). Caspase 1 activation in *Salmonella*-infected macrophages results in the production of active IL-1β and IL-18 and rapid cell lysis with the release of proinflammatory intracellular contents [58]. Knodler *et al.* [59] showed that *S. enterica* serovar *typhimurium* effector protein SopB protects epithelial cells from apoptosis by sustained the activation of Akt. SopB has antiapoptotic activity in infected epithelial cells, which is dependent on the phosphatase activity of SopB and the presence of Akt. PI3K-Akt/PKB-pathway protect against apoptosis by the phosphorylation of the proapoptotic Bad. This pathway prevents cytochrome *c* release.

### 6.5. *Listeria monocytogenes*

*Listeria monocytogenes*, a facultative anaerobe and/or intracellular bacterium, is the causative agent of listeriosis. *L. monocytogenes*, similar to *Shigella*, lyses the phagosomal membrane and escapes into the cytosol to initiate an intracellular infection, a process that is mediated by a secreted pore-forming toxin, listeriolysin O (LlyO). *L. monocytogenes* induces LlyO-dependent apoptosis in a variety of cell types, including hepatocytes, lymphocytes, and dendritic cells. LlyO might insert into the mitochondrial membrane causing the release of cytochrome *c*. In addition, the insertion of LlyO into the mitochondrial and/or endoplasmic reticulum membrane may stimulate calcium efflux, thereby activating the calpain and/or caspases [60,61]. Stavru and Cossart [62] showed that LlyO is responsible for mitochondrial network disruption along with a decrease in mitochondrial membrane potential and intracellular ATP levels. However, *L. monocytogenes* does not induce apoptosis in macrophages but causes LlyO-mediated necrosis [63].

### 6.6. *Chlamydiae*

Two species of chlamydiae commonly infect humans, *Chlamydia trachomatis* and *Chlamydia pneumoniae. C. trachomatis* causes trachoma, a scarring eye infection in developing countries. *C. pneumoniae* causes pneumonia [64]. *Chlamydia* species require differentiation to produce sufficient infectious elementary bodies to spread to adjacent cells. In an infection, elementary bodies are taken up by a host cell and begin their cycle inside a membrane-bound vacuole in the host cell cytosol. During the early stages of infection, *Chlamydia* has antiapoptotic effect that helps to maintain the metabolic activities of the infected cell. It was shown that *C. trachomatis* has antiapoptotic effect in epithelial cells and macrophages by blocking the release of cytochrome *c* [65]. *C. pneumoniae* also inhibited apoptosis through an additional activity that was described as blocking caspase activation by cytochrome *c* in a cell-free system [66]. The activation of the host cell apoptotic pathways in the late stages of infection may facilitate dispersal of the bacteria and initiate a host inflammatory response. The various subfamilies of the Bcl-2-family of proteins play a decisive role in this event. The trigger of cytochrome *c*-release is the activation of one or several BH3-only proteins. Bcl-2 and its antiapoptotic homologues can bind active BH3-only proteins and probably by this interaction block apoptosis [67]. Du *et al.* [68] demonstrated another way of that inhibiting apoptosis involves the activation of Raf/MEK/ERK survival pathway.

### 6.7. *Yersinia*

*Yersinia* invades epithelial cells, fibroblasts, and M cells in mammalian cells *in vitro* and *in vivo* [69,70]. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are transmitted by fecal-oral route and cause gastrointestinal syndromes lymphadenitis and septicemia. *Yersinia pestis* is typically transmitted through the bite of an infected flea and causes bubonic and/or septicemic plague. Yersinia virulence plasmid encodes T3SS and 6 known effector proteins termed Yersinia outer proteins (Yops) [71]. The translocation of the effector molecule YopJ (*Y. pseudotuberculosis*), YopP (*Y. enterocolitica*), or YopJ$^{KIM}$ (*Y. pestis*) into the cells has been shown to rapidly activate apoptosis in macrophages and dendritic cells but not in human epithelial cells [72,73]. Yersinia YopJ/P represses the activation of ERK and the NFKB. More recent studies indicate that YopJ has acetyltransferase activity, acetylating Ser and Thr residues critical for the activation of IKK-B and ERK kinases in *Yersinia*-infected macrophages [74,75]. Also, the TLR4 has been shown to act as a potent inducer of apoptosis in macrophage [76]. YopJ$^{KIM}$ has two amino acid changes that give it an enhanced ability to inhibit survival signals in macrophages. The increased apoptosis may cause membrane permeability resulting in the efflux of $K^+$ and activation of caspase 1. It was suggested that caspase 1 activation is a normal outcome of a type of apoptosis that is triggered in naive macrophages by TLR4 signaling combination with pathogen interference with ERK and NF-κB pathways [77]. YopJ and/or YopP did not induce pronounced apoptosis in human PMLs. It was suggested that Yersinia inhibition of $PML_S$ ROS production plays a role in evasion of the human innate immune response in part by limiting PMLs apoptosis [78].

### 6.8. *Legionella*

*Legionella pneumophila* invades and replicates within alveolar macrophages, monocytes, and possibly alveolar epithelial cells and causes Legionnaires disease. The expression and/or export of apoptosis-inducing factor(s) in *L. pneumophila* is regulated by the Dot/Icm type IV-like secretion system [79,80].

*L. pneumophila* utilizes several strategies to ensure intracellular replication and protect itself against the host immune system. These are the following: (1) upon entry into a human phagocyte, *L. pneumophila* becomes contained in a vacuole called *Legionella*-containing phagosome that avoids the typical fusion with the lysosome. *L. pneumophila* promotes the cleavage of Rabaptin-5 by caspase 3, thus preventing the default phagosome-lysosome fusion. (2) *L. pneumophila* promotes the cleavage of Rabaptin-5 by caspase 3, thus preventing the default phagosome-lysosome fusion. (3) *L. pneumophila* does not activate caspases 1 and 7 in human monocytes consequently aborting the phagosome-lysosome fusion. (4) *L. pneumophila* inhibits host cell apoptosis by upregulating antiapoptotic genes. (5) *L. pneumophila* controls the local balance of activating cytokines (IFN-$\gamma$ and/or TNF-$\alpha$) that inhibit its replication and inhibiting cytokines (IL-10) that allow its survival. (6) *L. pneumophila* activates the NF-κB pathway to maintain host cell survival and/or 7-*L. pneumophila* modulates other innate immune responses to establish a replicative niche [81].

### 6.9. *Escherichia coli*

*E. coli* K1 is a leading causative agent of neonatal meningitis. OmpA of *E. coli* can directly interact with monocytes and macrophages for entry. Some *E. coli* serotypes produce a Shiga-like toxin that can bind to human intestinal epithelium and produce Shiga-like toxins, which are associated with hemorrhagic colitis and the hemolytic-uremic syndrome [82]. One pathway involving apoptosis is mediated by death receptors such as CD95 and TNF-R. Intrinsic apoptotic pathway is involved in the Shiga-like toxin-mediated apoptosis of epithelial cells [83]. *E. coli K1* induces the expression of the antiapoptotic protein $Bcl_{XL}$ for its own survival and that of host macrophages. In addition, the bacteria may also block the activation of caspases. Besides upregulating $Bcl_{XL}$, $OmpA^{+}$ *E. coli* interaction with macrophages may alter the signaling pathways of the host cell to use it as a protected reservoir for the time required to reach septic levels [84,85].

### 6.10. *Rickettsia*

*Rickettsia* is a genus of nonmotile, Gram-negative, non-spore-forming, and/or highly pleomorphic bacteria. *Rickettsia rickettsii* is a unicellular Gram-negative coccobacillus. *R. rickettsii is* most commonly known as the causative agent of Rocky Mountain spotted fever [86]. *R. rickettsii* prevents apoptosis by the activation of cell survival pathways. *R. rickettsii* induces NF-κB activity and the upregulation of prosurvival proteins, the downregulation of proapoptotic proteins, and a lack of cytochrome *c* release in endothelial cells. Bechelli *et al.* [87] performed a screening of pro- and antiapoptotic genes that were differentially expressed in human microvascular endothelial cells during *R. rickettsii* infection and after staurosporine challenge.

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A total of 14 genes were significantly upregulated of which 8 (TRAF1, BNIP2, BCL2L1, TRAF3, BIRC2, BNIP3L, AKT1, and BIRC5) are known apoptosis suppressors while 6 are known to promote apoptosis (BOK, BCL2L13, DAPK2, TP53, ABL1, and BAK1). However, *R. rickettsii* efficiently infects neuronal cells and that the infection causes apoptotic death of neuron [88].

### 6.11. *Mycobacterium*

*Mycobacterium tuberculosis* is the causative agent of most cases of tuberculosis. *M. tuberculosis* infected macrophages die by (a) necrosis, a death modality defined by cell lysis, or (b) apoptosis, a form of death that maintains an intact plasma membrane. Necrosis is a mechanism used by bacteria to exit the macrophage, evade host defenses, and spread. *M. tuberculosis* is complex and includes both the induction of cell-death and cell-survival signals. It induces apoptosis in macrophages *in vitro* and *in vivo* [89]. Apoptosis occurs by TNF pathway. In addition, MOMP leading to the activation of the intrinsic apoptotic pathway is required. Both pathways lead to caspase 3 activation which then results in apoptosis [90]. Bacteria cell wall components connect TLR-2 molecules. *M. tuberculosis* also protects cells against apoptosis via two key pathways: first, through the induction of TLR-2-dependent activation of the NF-κB cell survival pathway, and second, by enhancing the production of soluble TNFR-2 (sTNFR2), which neutralizes the proapoptotic activity of TNF-$\alpha$. *M. tuberculosis* can prevent apoptosis in alveolar epithelial cells. Danelishvili *et al.* [91] showed that *M. tuberculosis* infection of macrophages results in the downregulation of the Bcl-2 gene and the upregulation of Bax and Bad proapoptotic genes. In contrast, the increased expression of Bcl-2 and the inhibition of Bax and Bad genes were observed in alveolar epithelial cells. *M. tuberculosis* infection was associated with the repression of the Bcl-2 gene and the induction of p53 in human macrophages. In alveolar epithelial cells, the expression of p53 was unchanged during *M. tuberculosis* infection [92].

## 7. Viral infections

After infecting target cells, viruses replicate to produce large number of progeny virions and spread the progeny to initiate the next round of infection. Some viruses encode specific proteins to optimize their replication. Infection by viruses, however, triggers the apoptosis of the infected cell to restrict virus infection. This is done by reprogramming of the host cell apoptotic pathway to effect death of the infected host cell before the release of progeny viruses. In order to ablate host defense mechanisms, viruses have evolved proteins that are able to inhibit or delay the host protective actions by targeting strategic points in the apoptotic pathways [93]. Apoptosis can be induced by intrinsic or extrinsic signals (Table 1). Intrinsic signals may result from viral infection and include stress, cell cycle arrest, cytoplasmic calcium perturbation, and DNA damage. Extrinsic signals arise as a result of the host immune response through TNF-receptor or Fas activation or via delivery of proteases by cytotoxic lymphocytes. Once induced, apoptosis may eliminate infected cells prior to release of viral progeny [94, 95]. Many viruses have been shown to induce apoptosis, either as a mechanism for the release and dissemination of progeny virions or as a defense strategy of multicellular host organisms for the destruction

of infected cells and therefore preventing the spread of the virus [96]. Innate and the acquired immune system induce apoptosis as a host defense against viral infections. The innate immune system directly activates inflammatory cells such as macrophages (e.g., granulocytes, Kupffer cells in the liver) and natural killer (NK) cells, which may directly cause death of the infected cells. On the other hand, viral RNA or proteins can bind to intracellular molecules that modulate or directly induce cell death [97]. In this immune cell-independent, virus-induced apoptosis of the host cell protein kinase R (PKR) and the cytoplasmic RNA helicase RIG-I play important roles [98]. PKR acts via the downstream transcription factor eIF-2$\alpha$ [99]. At the same time, acquired immune system works to eliminate the virus and the recognition of viral antigens presented by specific cells (e.g., dendritic cells). The antigen-primed $CD8^+$-T-lymphocytes cytotoxic T lymphocytes directly kill infected cells via direct cell-cell contact and release of cytotoxic and/or antiviral cytokines (e.g., interferons IFNs and/or TNF-$\alpha$), whereas IFN-$\gamma$ and IFN-$\alpha$ are also able to eliminate the virus without killing the host cell [100].

The IFNs are considered to play a critical role in innate immunity to viral infection and aside from effectively preventing intracellular viral replication can also mediate the activation and recruitment of the adaptive immune response. The IFNs can be induced by a number of stimuli, including viruses and dsRNA through mechanisms involving the activation of interferon regulatory factor (IRF-3), NF-κB, and perhaps the dsRNA-dependent PKR and the JNK2 pathway, all of which have been reported to be mediators of cell death [101,102].

### 7.1. Hepatitis C virus

Hepatitis C virus (HCV) causes liver cirrhosis and hepatocellular carcinoma [103]. In hepatocytes, apoptosis induction via cytotoxic T lymphocytes and macrophages largely occurs via extrinsic pathway. Ligand binding activates caspase 8 signaling cascade [104]. Another mechanism of apoptosis involves viral protein and their interactions. HCV core protein has been shown to be proapoptotic and antiapoptotic effects [105]. Machida *et al*. [106] showed that the expression of HCV proteins may directly or indirectly inhibit Fas-mediated apoptosis and death in mice by repressing the release of cytochrome *c* from mitochondria.

### 7.2. HIV

Human immunodeficiency virus type 1 (HIV-1)-infected individuals often suffer from neurological complications such as memory loss, mental slowing, and gait disturbance [107]. HIV infection is associated with a progressive decrease in and/or loss of $CD4^+$ and the decline of $CD8^+$ T cells and viral replication [108]. Inappropriate signaling through the binding of the HIV-1 envelope to the CD4 may induce abnormal programmed $CD4^+$ T-cell death. Viral proteins such as HIV-1 gp120 have an important role development in HIV-associated apoptosis. HIV proteins implicated in the induction of apoptosis *in vitro* include tat, nef, vpr, and protease. Cross-linking of bound gp120 on human $CD4^+$ T cells followed by signaling through the T-cell receptor for antigen was found to increase susceptibility to Fas and result in apoptosis [109,110]. In addition, deregulation in cytokine production occurs during HIV infection, perturbing the immune response. The overproduction of IL-4 and/or IL-10 cytokines is known to increase susceptibility to activation-induced cell death. IFN-$\alpha$ produced by HIV-1-infected

dendritic cells contributes to CD4 T-cell apoptosis by the TRAIL/DR5 pathway [111]. HIV-1 protein Env triggers apoptosis by the transactivation of the p53-dependent genes Puma and Bax [112]. HIV Nef is able to induce apoptosis by extrinsic pathway [113,114].

### 7.3. Rabies virus

Rabies virus (RV) is a neurotropic virus and travels to the brain by following the peripheral nerves. RV, a member of the genus *Lyssavirus* of the family Rhabdoviridae, is known to cause fatal encephalomyelitis in many mammalian species. RV has developed two main mechanisms to escape the host defenses: (1) its ability to kill protective migrating T cells and (2) its ability to sneak into the nervous system without triggering the apoptosis of the infected neurons and preserving the integrity of neurites [115]. In one of the studies, Ubol *et al.* [116] showed the expression of Bax and caspase 1 activation in RV-infected neuroblastoma cells. In another study, the expression of caspase gene Nedd-2 was significantly upregulated in infected adult and suckling mice [117]. Thoulouze *et al.* [118] showed that apoptosis induced by rabies virus involves the activation of caspase 8 and disappearance of procaspases 9 and 3. In addition, AIF translocated from the cytoplasm to the nucleus, suggesting that caspase-independent pathway is also involved in RV-induced apoptosis. Sarmento *et al.* [119] showed that AIF, a caspase-independent apoptotic protein, was upregulated and translocated from the cytoplasm to the nucleus postinfection, suggesting that apoptosis induced by RV induces apoptosis by both the caspase-dependent and caspase-independent pathways.

### 7.4. Epstein-Barr virus

Infection with Epstein-Barr virus (EBV) is very common and usually occurs in childhood or early adulthood. In fact, up to 95% of people in the U.S. have been infected with EBV. EBV is the cause of infectious mononucleosis (also termed “mono”), an illness associated with fever, sore throat, swollen lymph nodes in the neck, and sometimes enlarged spleen. Less commonly, EBV can cause more serious disease. To establish a persistent latent infection, EBV must access the memory B-cell compartment and reside within long-lived peripheral B cells where few viral gene products are expressed in order to escape immune detection [120]. EBV encodes two viral Bcl-2 proteins, BHRF1 and BALF1, with apparently redundant functions. The viral BHRF1 gene expresses a Bcl-2 homologue protein that resembles Bcl-2 in its subcellular localization and capacity to enhance B-cell survival [121]. *In vitro*, EBV infects resting human B lymphocytes and transforms them into lymphoblastoid cell lines (LCLs). In LCLs, 11 so-called latent genes are consistently expressed. These are the EBV nuclear antigens EBNA1, EBNA2, EBNA-LP, EBNA3A, B, and/or C and the latent membrane proteins LMP1, LMP2A, and B and two noncoding RNAs [122]. LMP1 indirectly inhibits apoptosis by upregulating several cellular antiapoptotic genes presumably through the induction of the NF-κB pathway [123].

### 7.5. Baculovirus

Baculovirus antiapoptotic genes include p35, which encodes the most broadly acting caspase inhibitor protein known and IAP genes [124]. The baculovirus IAP blocks apoptosis induced by caspase activation. All viral IAPs (vIAPs) contain a carboxyl ring finger and a variable

number of highly conserved Cys⁄His motifs known as baculoviral IAP repeats (BIRs). The BIR domains bind directly to caspases and inhibit their proteolytic activity and molecules that contain an IAP-binding motif (second mitochondrial-derived activator of caspases and Omi) antagonize IAP function via binding to BIR motifs displacing IAP binding to caspases or by promoting their degradation. Therefore, vIAPs act downstream of mitochondria, inhibiting the activity of procaspase 9 and effector caspases 3 and 7 [125]. The antiapoptotic protein p35 from baculovirus is thought to prevent the suicidal response of infected insect cells by inhibiting caspases [126]. Zhou *et al.* [127] showed that purified recombinant p35 inhibits human caspases 1, 3, 6, 7, 8, and 10. There may be interaction of the baculovirus antiapoptotic protein p35 with caspases. Sah *et al.* [128] demonstrated the ability of the p35 gene to inhibit oxidative stress-induced apoptosis. Oxidative damage to cellular macromolecules such as nuclear and mitochondrial DNA and proteins caused by reactive oxygen species is considered to be of key importance in the aging process. The chain of oxidative reactions initiated by ROS eventually knocks down the crucial biomolecules, thereby driving the cellular machinery to undergo apoptosis via the activation of caspases, which ultimately brings about the execution of cell death. p35 is able to directly mop out free radicals and prevent cell death by also acting in an oxidant-dependent pathway at a very upstream step in the cascade of events associated with oxidative stress-induced apoptosis [129].

### 7.6. Human papillomavirus (HPV)

HPVs are small DNA viruses that are known to be the most common etiological agents in cervical cancer. HPVs are implicated in the mucosal and epithelial infections that may range from a benign lesion to a malignant carcinoma [130]. Recent studies have shown that 13 different types of HPV are associated with carcinogenesis. HPVs are DNA tumor viruses whose genome is organized in three regions: the early gene (E1 to E7), the late gene (L1 and L2) regions, and the upper regulatory region (URR). The late region units, L1 and L2, encode for viral capsid proteins during the late stages of virion assembly. E1 and E2 encode proteins that are vital for extrachromosomal DNA replication and the completion of the viral life cycle. The E4 protein plays an important role for the maturation and replication of the virus. The E5 in open reading frame (ORF) interacts with various transmembrane proteins like the receptors of the epidermal growth factor, platelet-derived growth factor β, and colony stimulating factor-1. E6 and E7 ORF encode for oncoproteins that allow replication of the virus and the immortalization and transformation of the cell that hosts the HPV DNA [131]. The HPV E2 regulates the transcription of E6/E7 and facilitates apoptosis via p53-dependent pathway in HeLa cells [132]. HPV E7 is involved with cell cycling and binds to the retinoblastoma tumor suppressor protein and related proteins and induces apoptosis in mouse lens [133]. The inactivation of p53 by E6 should lead to a reduction in cellular apoptosis. Numerous studies showed that E6 could in fact sensitize cells to apoptosis. HPV E6 induce the degradation of p53. E6 expression correlated with the prolonged expression of Bcl-2 reduces the elevation of Bax and loss of p53. Several studies have shown that E2 could also induce apoptosis independent of its effects on transcription of E6 and E7 [134-136]. Tan *et al.* [137] showed that HPV16 E6 RNA interference enhances cisplatin and death receptor-mediated apoptosis in human cervical carcinoma cells. Moreover, HPV-16 E6 was shown to bind TNF R1 and protect cells

from TNF-induced apoptosis in mouse fibroblasts and human histiocyte/monocyte and osteosarcoma cells. Caspase 3 and caspase 8 activation were significantly reduced in E6-expressing cells [138].

### 7.7. Adenovirus

Adenoviruses (Ads) were first described as the etiological agents isolated from human adenoids and respiratory secretions that cause spontaneous cytopathic effects in cultures of human cells. Adenovirus has evolved ways to commandeer host cell machinery for successful entry, viral DNA replication, and propagation of progeny virions. Adenoviral proteins interact with host-cell proteins to either exploit or inhibit cellular functions for the purpose of viral propagation. The Ad genome is a 36 kbp linear double-stranded DNA molecule that encodes five early transcription units (E1A, E1B, E2, E3, and E4), two delayed early units, and one major late unit that is processed to make five families of late mRNAs. The early genes are transcribed before viral DNA replication begins, and the late proteins are made following the onset of replication [139]. At least 51 serotypes have been distinguished based on resistance to neutralization by antibodies specific to other known serotypes. These are divided into six subgroups (A-F) based on hemagglutination patterns, oncogenicity, and genome homologies. The common subgroup C Ads, which include Ad serotypes 1, 2, 5, and 6, are endemic virtually all over the world. They cause mild upper respiratory tract infections in young children [140]. Early in infection, the expression of E1A drives the host cell into the S phase of the cell cycle in order to induce DNA synthesis that is required for viral replication. The genes in the E3 region of Ad encode several proteins that function to protect the virus-infected cell from host immune responses [141]. Table 1 describes the adenovirus immunoregulatory proteins and how they function to block or induce apoptosis of infected cells [142-150].

### 7.8. Human cytomegalovirus

Human cytomegalovirus (CMV), a beta herpes virus with a widespread distribution, is a major cause of morbidity and mortality in immunocompromised individuals such as organ transplant recipients and patients with AIDS. During pregnancy, CMV is a major cause of congenital disease [151]. CMV genes UL36 and UL37 encode viral inhibitor of caspase-8-induced apoptosis (vICA) and viral mitochondria inhibitor of apoptosis (vMIA), respectively. Skaletskaya *et al.* [152] identified a human cytomegalovirus cell-death suppressor denoted vICA and encoded by the viral UL36 gene. vICA inhibits Fas-mediated apoptosis by binding to the prodomain of caspase 8 and preventing its activation. vMIA blocks cytochrome *c* release and activation of downstream effector caspases in a manner analogous to Bcl-2 homologues. Like Bcl-2, vMIA localizes to mitochondria and inhibits mitochondrial permeabilization induced by apoptotic signals [153]. vMIA also counteracts serine protease HtrA2/Omi (high temperature requirement protein A2/Omi stress-regulated endonuclease)-dependent cell death and allows infected cells to survive and continuously produce a virus for several days [154]. Additional human CMV gene products, including IE1 and IE2, as well as the murine CMV UL45 homologue may influence cell susceptibility to apoptosis [155]. Transient transfection assays indicate that the IE1 and IE2 proteins regulate transcription. The IE1 and IE2 proteins

each inhibit the induction of apoptosis by TNF or by the E1B 19-kDa-protein-deficient adenovirus. IE1 and IE2 proteins inhibit apoptosis in part by modulating the activity of p53[156]. In addition, IE1 and IE2 and the viral RNA beta 2.7, which bind to the mitochondrial respiratory complex I, maintain ATP production late in infection and prevent death induced by mitochondrial poison [157].

## 8. Apoptosis and parasitic infections

Apoptosis plays crucial roles in the interaction between the host and the parasite. This includes innate and adaptive defense mechanisms to restrict intracellular parasite replication as well as regulatory functions to modulate the host's immune response. During their evolution, parasites have developed mechanisms to induce or avoid host cell apoptosis in order to be able to survive and complete their life cycle (Table 1). Among the factors involved in that balance in infected organisms, the time of apoptosis (early or late occurrence), the cell type, and the type of parasitism (intracellular or not) are the major modulators. For example, the early apoptosis of host cells could contribute toward their fight against infection by intracellular parasites; equally, early apoptosis could favor the penetration of the parasite. The late apoptosis of cells of the defense system could be beneficial to the host clearing excess cells, thereby avoiding the detrimental effects of excessive inflammatory response in the tissue that they would cause [158].

### 8.1. *Toxoplasma gondii*

*Toxoplasma gondii* is a species of parasitic protozoa in the genus *Toxoplasma*. Humans can become infected with *T. gondii*, either through contact with soil contaminated by cat feces or by eating infected meat. Toxoplasmosis is usually asymptomatic because our immune system keeps the parasite from causing illness. The disease is more problematic for pregnant women and people who have weakened immune systems. Some results indicate a strong correlation between schizophrenia, brain cancer, and toxoplasmosis [159]. Toxoplasma promote or inhibit apoptosis. Begum-Haque *et al.* [160] demonstrated marked difference in the death of activated T cells between early (day 3 post infection) and acute (day 6 post infection) stage of *T. gondii*. The decreased production of IL-2 and augmented synthesis of IL-10 during acute stage of *T. gondii* infection may have a role in the enhanced level of apoptosis. It has been suggested that the apoptosis of T lymphocytes in *T. gondii* infection is associated with the virulence and density of the parasite in the host. In *T. gondii* infection, IFN-$\gamma$ locally produced in Peyer's patches contributes to the induction of apoptosis in Peyer's patch T cells [160]. *T. gondii* inhibits the apoptosis of host cells by indirect and direct mechanisms. Granulocyte colony-stimulating factor and granulocyte-macrophage cerebrospinal fluid secreted by *T. gondii*-infected human fibroblasts increased the expression of antiapoptotic Bcl-2 family member Mcl-1 and abolished apoptosis in neutrophils *in vitro* indirectly [161]. Many studies have shown that *T. gondii* has evolved strategies to directly inhibit cell apoptosis by various mechanisms: (a) increased expression of antiapoptotic members of the Bcl-2 protein family; (b) inhibition of the cytochrome *c* release; (c) upregulation of $IAP_s$; (d) activation of NF-κB by *T. gondii* in distinct cell

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types or under distinct conditions thereby inducing the transcription of genes encoding antiapoptotic molecules, including Bfl-1 and IAPs; and/or (e) degradation of the PARP as described is involved in the inhibition of apoptosis. Although direct evidence is still lacking, it appears plausible that diminished PARP levels in *Toxoplasma*-infected cells may inhibit apoptosis in a caspase-independent fashion [162].

### 8.2. *Plasmodium falciparum*

*Plasmodium falciparum* is the agent of malaria. Enhanced levels of RBC apoptosis have been observed in clinical disorders in which anemia is a common feature such as iron and renal insufficiency, thalassemia, sickle-cell disease, and apoptosis has been associated to cerebral malaria, thrombocytopenia, and lymphocytopenia in malaria infection [163].

*P. falciparum* induces oxidative stress, which in turn activates the $Ca^{+2}$ permeable cation channels followed by $Ca^{+2}$ entry, and the stimulation of eryptosis has been coined to describe the suicidal erythrocyte death. The $Ca^{+2}$ uptake, however, eventually triggers eryptosis of the parasitized erythrocyte, and thus the parasitized erythrocytes is doomed to be phagocytosed by macrophages [164].

*P. falciparum* firstly enter red blood cells. Second, parasitized red blood cell sticks endothelial cells, inducing the expression of iNOS in brain cells. The activation of caspases 8 and 9 results in apoptosis and blood-brain barrier disruption [165].

### 8.3. Trypanosomatids

Trypanosomatids are the causative agents of diseases such as the Chagas disease and the African sleeping sickness [166]. Trypanosomatids lack some of the key molecules contributing to apoptosis in metazoans like caspase genes, Bcl-2 family genes, and the TNF-related family of receptors. Apoptosis triggered in response to heat shock, prostaglandins, antibodies, and mutations in cell cycle regulates genes [167]. These stimuli result in loss of $\Delta\Psi m$, generation of ROS, lipid peroxidation, and increase in cytosolic $Ca^{2+}$. This also potentiates the release of cytochrome *c* and EndoG into the cytoplasm and the activation of proteases and nucleases to dismantle the parasites in an ordered fashion. Upon release from the mitochondrion, EndoG translocates to the nucleus to degrade DNA. These events finally lead to the execution of apoptosis [168]. *Trypanosoma brucei* causes neuronal demyelination and apoptosis after blood-brain barrier damage. This leads to apoptosis in cells of the cerebellum and brain stem. Welburn *et al.* [170] described cytoplasmic vacuolization and marginalization, extensive membrane blebbing, and condensation of nuclear chromatin in *Trypanosoma cruzi* and *T. brucei* respectively. Lectins such as ConA were among the first compounds shown to induce the expression of apoptotic markers in *T. brucei* [169]. *T. cruzi* is the etiological agent of Chagas disease. It also inhibits apoptosis through the action of parasite-derived neurotrophic factor, a parasite-derived protein in neuronal and glial cells. The parasite-derived neurotrophic factor is both a substrate and an activator of the serine-threonine kinase Akt and an antiapoptotic molecule binding to the neurotrophic surface receptor TrkA (neurotrophic tyrosine kinase receptor type 1) triggering the PI3-K/PKB pathway resulting in increased Bcl-2 expression.

This results in protection of Schwann cells from apoptosis induced by $H_2O_2$ and TNF-$\alpha$/TGF-$\beta$ (transforming growth factor b) [170-172].

### 8.4. *Leishmania*

Leishmanias are agents of ulcerative skin lesions (cutaneous leishmaniasis) and disseminated visceral infection (visceral leishmaniasis or kala-azar). Leishmania is able to inhibit the spontaneous apoptosis of short-lived neutrophils, increasing their life span and providing a safe place for the parasites during the first days of the infection [173]. With most apoptosis inducing stimuli, *Leishmania donovani* shows typical features of apoptotic death like cell shrinkage, nuclear condensation, and DNA fragmentation. $Ca^{2+}$ appears to be a vital ion involved in Leishmania apoptosis. Extracellular or intracellular $Ca^{2+}$ during oxidative stress results in the significant rescue of the fall of the mitochondrial membrane potential and consequently apoptosis [174].

## 9. Concluding remarks

- Apoptosis is a genetically programmed process of cellular destruction that is indispensable for the normal development and homeostasis of multicellular organisms.
- Microorganisms induce apoptosis by intrinsic and extrinsic pathway in the host cell.
- T3SS effectors have also been shown to tamper with the host's cell cycle, and some of them are able to induce apoptosis bacteria such as *Pseudomonas*, *Shigella*, *Salmonella*, and *Yersinia*.
- Microorganisms inhibit apoptosis by multiple mechanisms: protection of the mitochondria and prevention of cytochrome *c* release (i.e., *Chylamidia* sp. and/or *Neisseria* sp.), activation of cell survival pathways (i.e., *Salmonella* sp. and/or *Rickettsia* sp.), inhibition of caspases, activation of phosphoinositide 3-kinase (PI3K)-Akt/protein kinase B (PKB) pathway, and interaction with cellular caspases (i.e., *Shigella* sp. and/or *Legionella* sp.)
- Prevention of apoptosis enables microorganisms to replicate and survive in host.
- A clear understanding of the molecular basis of apoptosis inhibition or induction is needed.
- Elucidation of the mechanisms, the cellular receptors, and/or the microbial factors involved in modulating of apoptosis could reveal insights into the host-pathogen relationship and new therapeutic targets.

## 10. Abbreviations

**AIF:** apoptosis inducing factor

**AP-1:** activator protein 1

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**BH:** Bcl-2 homology

**CARD:** caspase activation and recruitment domain

**CMV:** cytomegalovirus

**CTL:** cytotoxic T lymphocyte

**DD:** death domains

**DED:** death effector domain

**$\Delta\Psi_m$:** transmembrane potential

**EBV:** Epstein-Barr virus

**FADD:** Fas-associated death domain

**GSK-3:** Glycogen synthase kinase 3

**JNK:** c-Jun N-terminal kinase

**HIV-1:** human immunodeficiency virus type 1

**IAPs:** inhibitors of apoptosis proteins

**IL-1β:** interleukin 1β

**ICE:** IL-1β converting enzyme

**I-KB:** inhibitor of KB

**IKK:** I-KB kinase

**IPA:** invasion plasmid antigens

**LlyO:** listeriolysin O

**MOMP:** mitochondrial outer membrane permeabilization

**NF-κB:** nuclear factor-kappa B

**TLR:** toll-like receptors

**TNF:** tumor necrosis factor

**TNFR:** tumor necrosis factor receptor gene

**TRAIL:** tumor necrosis factor (TNF)-related apoptosis-inducing ligand

**T3SS :** type III secretion system

**PI3K:** phosphoinositide 3-kinase

**PKB:** protein Kinase B

**PMLs:** polymorphonuclear leukocytes

**proIL:** prointerleukin

**PT:** permeability transition

**ROS:** reactive oxygen species

**RV:** Rabies virus

**SAPK:** stress-activated protein kinase

**STS:** staurosporine

**VDAC:** voltage-dependent anionic channel

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Chapter 13

# Caspases as Putative Biomarkers of Cervical Cancer Development

Olga V. Kurmyshkina, Pavel I. Kovchur and Tatyana O. Volkova

Additional information is available at the end of the chapter

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**Abstract**

Resistance to apoptosis is commonly accepted as the principal hallmark of a cancer cell, while caspases are recognized as the key molecular players of the apoptosis regulatory network. Since the level of caspase activity is thought to be directly coupled with aggressive features of cancer cells (such as ability to withstand immune reactions, invasiveness, drug resistance, etc.), these proteases could serve as objective diagnostic markers especially for those types of cancer where early differential diagnosis is needed. Cervical cancer develops through morphologically well-described stages—from intraepithelial lesions of 1/2/3 grade including carcinoma *in situ* to microinvasive and invasive cancer with precancerous lesions known to be potentially reversible. The percentage of cervical neoplasms diagnosed at early stages is relatively high, providing a basis for the use of cervical cancer as an *in vivo* model to investigate the mechanisms of apoptosis modulation in malignant cells. The existing diagnostic criteria, despite their usefulness, have substantial limitations with respect to cervical cancer and preneoplastic lesions, so caspases may be helpful in improving them, but there is insufficient data regarding the involvement of these enzymes in cervical cancer development. In this chapter, we report on specific patterns of activity of caspases revealed in tissue biopsies and blood lymphocytes in association with different stages of cervical cancer development. The data indicate that caspases are pivotal components of the *in vivo* molecular "portrait" of cervical cancer and have the potential of being used as biomarkers.

**Keywords:** Biomarkers of carcinogenesis, apoptosis, caspases, cervical cancer, human papillomavirus

## 1. Introduction

Carcinogenesis of solid tumors is a complex multistage process that generally develops over a long period of time through a succession of precancerous lesions. Cancer *in situ* and micro-

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invasive cancer before the foci of tumor invasion and areas of metastatic growth are established. Until a certain step, the process is reversible—due to various intracellular control mechanisms and the immune system surveillance. Programmed cell death (PCD) represents the key mechanism for maintaining cellular homeostasis of a multicellular organism and eliminating abnormal potentially dangerous (transformed/virus-infected) cells that may be induced by either activation of the intracellular sensors of molecular abnormalities or by exposure to the cytotoxic factors secreted by immunocompetent cells. According to the concept of Hanahan and Weinberg [1], resistance to PCD inducers is a fundamental characteristic of a malignantly transformed cell. Various forms of PCD are presently well recognized with apoptosis still regarded as the major pathway. Apoptotic cell death is characterized by clearly defined morphological and biochemical changes and generally requires the involvement of a special class of intracellular proteases—the caspases (E.3.4.22).

In the process of carcinogenesis, the ability to inhibit apoptosis is believed to be established by gradual accumulation of mutations and/or epigenetic modifications of tumor suppressor genes with further selection of the most resistant cell clones. The expression levels of molecular components of the apoptotic signaling pathway and their functional activity reflect the degree of malignization at the site of a developing neoplasia. At the same time, in their research practice when studying the molecular mechanisms of apoptosis inhibition and searching for the ways of its reactivation, scientists usually deal with tumor cell lines and xenograft models that represent the final result of a long process of establishing the ability to resist apoptotic death and thus do not reproduce the complex multistep nature of its development to the full extent. The major reason for this situation lies in the fact that, for many types of cancer, the initial steps of carcinogenesis (precancer lesions, true noninvasive cancer, and microinvasive carcinoma) are very difficult or practically impossible to diagnose.

Cervical cancer (CC) exemplifies an oncopathology for which all the steps of carcinogenesis, including the earliest ones—cervical intraepithelial neoplasia (CIN) grades 1, 2, and 3 as well as microinvasive cancer— are described in sufficient detail relying on morphological criteria as opposed to many tumor types having other locations (Figure 1). Thanks to the successful implementation of the mass screening prevention programs, the percentage of cervical neoplasms diagnosed at early stages is notably higher than that of advanced stages representing another distinctive feature of CC. Precancerous (intraepithelial) lesions generally persist over a long period of time and, according to epidemiological evidence, undergo spontaneous regression with the high rate of frequency [2], but once a malignant phenotype is established, rapid invasion and fast dissemination are observed. Thus, the availability of a “set” of thoroughly defined and easily detectable stages of CC development gives enough reasons for considering CC as a unique *in vivo* model for studying the driving forces and the mechanisms of carcinogenesis, including pathways of PCD dysregulation. So far as the problem of PCD is concerned, the viral etiology of CC should be mentioned: human papillomavirus (HPV) is the cause of more than 99% of CC instances, and repression of both the differentiation program and apoptosis in an infected cell is thought to be the main way for the virus to implement its replicative strategy upon “productive infection,” the question to be discussed in more detail in the main body of the chapter.

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Not only understanding of the fundamental significance but also promising opportunities for investigation of new diagnostic criteria and therapeutic tools can explain a constantly increasing interest to the use of CIN and CC as a natural model for the study of the apoptosis dysregulation pathways. The broad resistance of tumor cells to inducers of apoptosis remains one of the largest (if not the largest) obstacles to the effective implementation of the most of approaches currently being developed for radio-/chemo-/immunotherapy of human malignancies. CC is one of the most aggressive types of cancer as it is characterized by rapid acquisition of chemoradioresistance, fast progression, quick dissemination, and high recurrence rate even if diagnosed at the earliest stages. Despite the fact that CC is frequently diagnosed at early (including preclinical) stages, it still has one of the highest mortality rates, indicating insufficient level of our knowledge of the apoptosis inhibitory mechanisms involved in CC development and acute need of searching for the novel diagnostic biomarkers.

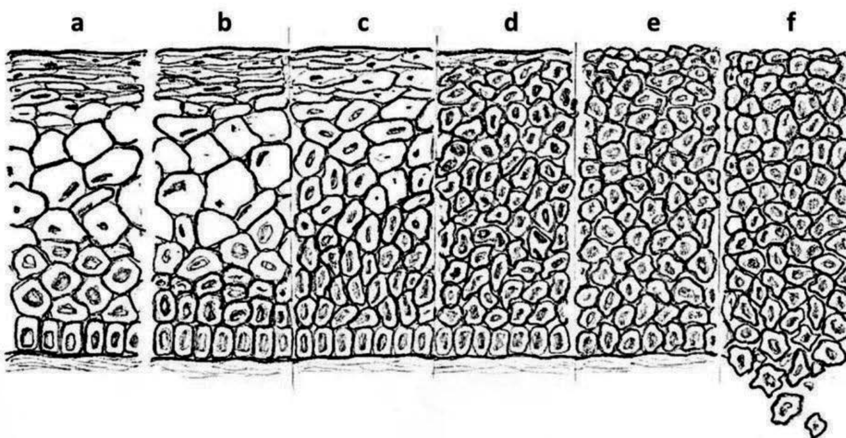


**Figure 1.** (a) Squamous cell epithelium of the cervix (the normal structure); (b–f) the continuum of cervical cancer development. Replication of HPV within the basal layer results in hyperplasia of parabasal cells encompassing no more than one-third of epithelium thickness and designated as CIN 1 (b). In CIN 2 (c), actively proliferating cells spread to the one-half of epithelial depth, while in CIN 3 (d–e), they constitute almost all the layers of cervical epithelium. Microinvasive cancer (f) is defined as a lesion in which neoplastic cells disrupt the basement membrane and invade the underlying stroma to a depth ≤3 mm.

Genome-wide studies have clearly demonstrated that cancerous cells, including cervical cancer cells, exploit a great variety of pathways in order to suppress or to bypass apoptosis. An important inference that can be drawn from these studies is that either the survival of a tumor cell or its death does not depend on the expression level of individual pro- and antiapoptotic factors but is rather determined by the ratio of activities of multiple apoptosis regulators (both activators and inhibitors), i.e., "molecular context." Caspases are those molecular players that eventually read the context, thereby implementing apoptosis-resistant or apoptosis-sensitive phenotype of a cell. On the one hand, caspases have been the central subject of research in the field of molecular oncology for many years but, on the other hand, analyzing the articles discussing the role and functional features of caspases in the natural

history of CIN and CC, one can come to a surprising conclusion that there is little information on this issue. The available data are, to some extent, conflicting and related, for the most part, to the artificial cell systems in which the level of caspases' activity/expression is often considered just as an indicator of experimental exposures. That is the reason why we have focused our attention on investigation of expression and functional activity of caspases at different steps of CC development. In the present chapter, we aimed to prove, relying on both our observations and published data, that caspases are the central targets for the action of antiapoptotic mechanisms driving the process of CC development. We also tried to show that the caspases activity pattern and the level of apoptosis in the fraction of blood lymphocytes may represent a valuable characteristic for estimating a patient's immune status in its response to different treatment approaches. Taking into account the specificity of changes revealed for different caspases and different steps of CC progression discussed in the chapter, we propose to consider caspases as promising biomarkers for cervical malignancies.

## 2. Body

### 2.1. Molecular mechanisms of cervical cancer development—interference of HPV oncogenes with apoptotic program of an infected cell

As was mentioned above, cervical cancer originates from persistent papillomavirus infection. In most cases, CC development is associated with the so-called high-risk HPV types, with HPV16 and HPV18 being the most common variants. The HPV genome has a small size and codes for several proteins, three of which—E6, E7, and E5—being regarded as proteins with oncogenic properties in view of their capacity to stimulate cell proliferation, convert cells into immortalized state, increase the frequency of mutations, and suppress apoptosis.

HPV displays a clearly defined tropism in relation to proliferating cells of the basal layer of squamous cervical epithelium since it is in these cells that the DNA replication "machinery" required for the virus to replicate itself is maintained in the active state. As basal keratinocytes move from the growth layer to the upper ones, they normally exit cell cycle and undergo terminal differentiation; however, this might impose limitations on the replicative potential of the virus. To evade this, the HPV oncoproteins provide conditions that enable infected cells to retain the ability to perform DNA synthesis even in the highly differentiated state ("productive HPV cycle"). The aberrant initiation of S-phase can occur owing to the E7-mediated degradation of pRb-protein releasing E2F transcription factor that triggers the expression of cyclins and other S-phase regulators. At the same time, in order to avoid p53-dependent apoptosis promoted by the uncontrolled cell cycle activation, E6 protein targets p53 for proteasomal degradation. Thus, the inhibition of apoptosis is an integral component of the HPV life cycle, which nevertheless does not lead to malignant transformation since proliferative and antiapoptotic activity of E6 and E7 is overcome by mechanisms of negative control of cell division [3]. At the morphological level, the progression of productive infection manifests itself in the form of hyperplasia of the deepest layers of epithelium, referred to as CIN 1 (see Figure 1). However, sometimes the HPV life cycle may take the path of "nonproductive infection,"

followed by the overproduction of only two of the viral proteins, E6 and E7, but not resulting in the release of mature viral particles. An accidental transition of HPV from episomal state to the integrated one (happening presumably at CIN2/3 stage) is commonly accepted to be the main cause of dysregulation of the viral genome expression; however, the involvement of other mechanisms (e.g., epigenetic) is assumed as well. Anyway, the nonproductive infection can be considered as a by-product of HPV activity that significantly increases the probability of malignant transformation of cervical keratinocytes.

The mechanism of E6/E7-induced transformation is not confined exclusively to the degradation of the key cellular “guardians” pRb and p53. Recent studies have elucidated the existence of an intricate HPV interactome, i.e., a network of intermolecular interactions of E6 and E7 with the host cell proteins [4]. By these interactions, the E6 and E7 proteins can dramatically change the profile of gene activity, epigenomic landscape, and host cell proteome and modulate the majority of intracellular signaling pathways (including MAPK-, Wnt-, Akt-, Notch-, mTORC-, and STAT-dependent cascades), leading to virtually total reprogramming of an epithelial cell [2]. Degrading the p53 и pRb tumor suppressors, E6 and E7 remove cell cycle “checkpoints,” favoring the gradual accumulation of somatic mutations. Moreover, E7 can impair the formation of the mitotic spindle causing large-scale chromosome aberrations and inducing genomic instability. At the phenotypic level, all of these molecular alterations facilitate acquisition of the properties referred to as “The 10 Hallmarks of Cancer” [1, 3], with the resistance to the programmed cell death, or apoptosis, being the basic one.

Molecular mechanisms of apoptosis have been thoroughly investigated in recent decades, with new endogenic factors exhibiting pro- and antiapoptotic activity being constantly discovered. A wide diversity of factors and receptors inducing apoptosis reactions in a cell were identified; however, almost all of the signaling paths were shown to converge, one way or another, to the common effector molecules—caspases. Caspases are a family of intracellular cysteine-dependent endoproteases produced in the form of nonactive proenzymes and, in case of receiving an appropriate signal, activated on the cascade principle. Eleven human caspases are presently known. The activation of caspases can proceed by the two major mechanisms: (1) oligomerization on specific “molecular platforms” and (2) partial proteolytic cleavage. The first mode of activation is typical for the so-called initiator caspases, which include caspases 2, 8, 9, and 10. The initiator caspases can accept extra- or intracellular signals and trigger “extrinsic” or “intrinsic” apoptotic pathway, respectively. The extrinsic pathway generally starts with the activation of a cell death receptor: binding of the receptor extracellular domain to its ligand brings its cytoplasmic part to the conformational change that then triggers the assembly of a membrane-associated macromolecular signaling complex (DISC). DISC serves as a “platform” for the recruitment and activation of the caspase 8 or 10 multimers. Several cell death receptors belonging to the Tumor Necrosis Factor (TNF) superfamily, namely, Fas/CD95/APO-1 (a prototypic member), TNF-R1, TRAIL-R1, TRAIL-R2, DR3, DR4, DR5, and DR6, have been discovered to date. In case of the intrinsic pathway of apoptosis, the apoptosome is such a molecular “platform” that supports the process of caspase 9 activation. The apoptosome complex assembly can be initiated in response to metabolic stress, disturbance of mitochondrial membrane integrity, or DNA damage. When activated, initiator caspases perform

processing of the downstream executioner (effector) caspases 3, 6, and 7, which, in turn, destroy numerous structural, regulatory, and catalytic intracellular proteins. Enzymatic activity of caspases is tightly regulated by various endogenic inhibitors and activators, including IAP-1/-2, Bcl-2 family members, Smac/DIABLO, c-FLIP, Survivin/BIRC5, XIAP, NAIP, livin, and others. The level of activity of caspases (and thereby susceptibility to apoptotic signals) is thought to be determined by the ratio of expression levels of their endogenous modulators. Many human diseases, including neoplasms, are known to be accompanied by the repression of caspases functions; however, this repression usually not associated with abnormalities in their gene structure that distinguishes caspases from the so-called tumor suppressors. Inactivation of caspases occurs in cancer cells as a consequence of the overexpression of their inhibitors or as a result of suppression of the upstream components of the apoptotic signaling pathways, as for example death receptors [5].

Due to active mutational processes and viral etiology, cervical cancer is characterized by particularly early and rapid development of resistance to apoptosis. Numerous articles reporting on the influence of different variants of post-transcriptional E6/E7 silencing on the cell sensitivity to the apoptosis inducers provide strong evidence that hyperexpression of HPV oncogenes is the primary cause and a prerequisite for the development and maintenance of apoptotic-resistant phenotype. For example, the transfection of E6-siRNAs (small interfering RNAs) into CC cells conferred susceptibility to cisplatin-induced apoptosis [6]. Similarly, the expression of an E7-targeted RNA-aptamer disrupting the interaction between E7 and pRb resulted in the induction of apoptosis [7]. Treatment with the synthetic peptide anti-E7 antagonist was shown to suppress tumor growth in an animal xenograft model due to activation of apoptotic cell death [8]. Analysis of the published data revealed three basic groups of mechanisms that modulate apoptosis signaling pathways by engagement of HPV oncoproteins: (1) inactivation of proapoptotic proteins resulting from direct binding to E6/E7 with subsequent ubiquitination and proteasome-mediated degradation; (2) interactions of E6/E7 proteins with the cellular transcription factors and chromatin-remodeling enzymes leading to the change of either the mRNA expression pattern of pro- and antiapoptotic factors, or the profile of microRNAs (miRNAs) targeted these factors; and (3) HPV oncogene-mediated induction of genomic instability that causes either the accumulation of inactivating mutations in the proapoptotic oncosuppressor genes, or, alternatively, the amplification of antiapoptotic genes.

1. "High-risk" HPV oncoproteins are capable of high-affinity binding to various protein components of the extrinsic or intrinsic apoptosis pathways and stimulating their degradation due to the ubiquitin-ligase activity, thereby blocking signal transduction from an apoptogenic stimulus. In HPV-positive cells, the membrane expression of CD95/Fas, the key cell death receptor, is significantly reduced, and the DISC assembling is impaired because of accelerated destruction of the FADD adaptor protein and caspase 8, with endogenous inducers of mitochondrial apoptosis pathway (such as Bid, Bak, and Bax antagonists of Bcl-2 protein) being degraded as well (for a review, see [9, 10]). That is why the abrogation of proteasome functions (by MG132 or Bortezomib treatment, for example) potentiates the activity of caspases and sensitizes CC cells to TRAIL-/Fas-dependent apoptosis or radiation-induced cell death [11]. E5 protein, similarly to E6 and E7, can impair the mechanisms of CD95L- and TRAIL-mediated apoptosis [10, 12].

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2. Epigenetic modification of apoptotic genes is one of the mechanisms of global regulation of the cell death program in cervical cancer. HPV has been shown to dramatically alter the host DNA methylation landscape, especially within the promoter regions of tumor suppressor genes and genes coding for apoptosis activator proteins, thus facilitating conversion of these genes into the heterochromatin state. For example, the promoter hypermethylation of PRDM14 gene encoding a transcription factor which is required for the expression of NOXA and PUMA proapoptotic regulators of Bcl2-family was observed in HPV16-bearing cell lines and primary tumors [13]. Histone acetylation/deacetylation is another way of modulating gene activity employed by CC cells. The treatment of CC cell lines with various histone deacetylase inhibitors results in apoptosis induction followed by activation of caspases 3, 8, and 9, PARP cleavage, and loss of mitochondrial membrane potential, thus confirming the importance of this epigenetic mechanism for the establishment of apoptotic resistance [14–16]. In a similar manner, silencing of the MLL5β histone methyltransferase has an apoptosis-inducing effect on CC cells [17]. Transcriptomic studies also suggest that among the different functional groups of genes whose expression is affected by the presence of HPV oncogenes, the apoptosis-regulatory genes (as for example, BCL2, BCLXL, and c-IAP1 [18]) constitute a substantial portion. The expression profile of pro- and antiapoptotic miRNAs in CC cells also arouses much interest among researchers. More than 100 miRNA species were documented to change their expression in the presence of the viral E6/E7 proteins, and many of their mRNA-targets were found to code for various regulators of apoptosis and, in particular, caspases, as for example survivin and Bcl-family proteins [19–22]. The novel long noncoding RNAs (lncRNAs) contributing to the development of apoptosis-resistant phenotype of CC cells have been described as well [23].

3. In cervical cancer cells, the deletions of the chromosome loci containing genes required for the apoptotic program to be implemented are found to occur at a high frequency [24, 25]. Although having sporadic nature of occurrence, these genetic abnormalities most likely confer a selective advantage to cancer cells for further expansion that probably explains why the incidence of such abnormalities increases with tumor progression [24]. In contrast, for genes encoding inhibitors of apoptosis, amplification of the corresponding genome segments is frequently observed with CC progression [26].

As evidenced by the above-stated examples, the mechanisms cervical cancer cells employ to achieve apoptotic resistance engage all the levels of intracellular regulation—genomic, transcriptomic, epigenomic, and proteomic. Although all the diversity of the known molecular pathways is ultimately directed to the suppression of caspases as the crucial mediators of cell death reactions, the experimental data showing that their proteolytic activity does undergo specific changes in the natural history of cervical neoplasms appeared to be virtually absent in literature, thus prompting us to conduct research whose results are described below.

### 2.2. Induction of apoptosis in immunocompetent cells as a putative factor of cervical cancer progression

Because of the viral etiology of cervical cancer, the mechanisms of its development need to be investigated in conjunction with changes occurring in the immune system. In each individual

case, it is not only the properties of tumor cells (the mutation spectrum, the gene expression profile) that determine the progression of CIN to invasive metastatic state but also the survival and growth of secondary tumor foci. The abilities of the immune system to recognize and eliminate virus-infected and malignantly transformed cells are believed to be of great importance too. According to the general conception, it is due to the reactions of the immune system that both the HPV infection and dysplastic alterations of squamous epithelium (CIN1/2) are usually transient, and there is only a small percent of cases that develop to chronic or malignant form [2]. On the other hand, various mechanisms exploited by the virus and/or tumor cells for specific inhibition or avoidance of immune reactions are becoming elucidated in the last years. Furthermore, possible involvement of supplementary "environmental" factors exerting suppressive influence on the immune system of an organism is assumed. The induction of apoptosis in immunocompetent cells is supposed to be one of the mechanisms to inhibit antitumor/antiviral immunity. Although there is some evidence of increased expression of apoptosis-related markers in tumor-infiltrating or circulating lymphocytes of cancer patients, in case of cervical cancer, such information is scarce [27, 28]. CC cell lines as well as primary CC cells are known to express CD95L (FasL) [29] and, when cocultured, to induce apoptotic death in cytotoxic T-lymphocytes, the effect being abolished by anti-CD95 antibodies [30]. It was also found that treatment of peripheral blood lymphocytes taken from healthy donors with conditioned media from CC cell lines could induce apoptosis in subpopulation of CD4+ T-helpers [31]. Reasoning from these facts, we hypothesized that during its *in vivo* development cervical cancer may withstand immune reactions via promoting apoptosis in effector immune cells, and specific change of activity/expression of caspase may therefore be detected in circulating lymphocytes of CC patients.

### 2.3. Materials and methods

#### *2.3.1. Patients and samples*

Tissue samples and peripheral blood were obtained from 156 patients who underwent surgery in Oncological Dispensary of the Republic of Karelia: 75 women diagnosed with cervical intraepithelial neoplasia grade 3 (CIN 3, with average age at diagnosis 32.9 ± 7.4 years) and 81 women with squamous carcinoma, including 45 with stage IA (average age 31.3 ± 6.0), 21 with stage II (average age 43.6 ± 13.2), and 15 with stages III–IV (average age 46.9 ± 11.1), were examined. Stages of cancer were defined in accordance with the TNM-classification and the International Federation of Gynecology and Obstetrics system (FIGO, 1994). CIN 3 and CC diagnosis was based on comprehensive physical examination, extended colposcopy findings, cytology, and histopathology tests, in full compliance with the approved standards for the diagnosis and treatment of patients with gynecological malignancies. All women enrolled in this study were informed and gave voluntary written consent. The research was approved by the Committee on Medical Ethics of Petrozavodsk State University and the Ministry of Healthcare and Social Development of the Republic of Karelia.

Extended colposcopy was performed in each case before surgery in order to define localization and precise margins of a lesion for subsequent accurate excision of tissue fragments.

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Colposcopic findings were evaluated according to the International Federation for Cervical Pathology and Colposcopy (IFCPC, 2002) terminology. Tissue samples were obtained during cervical conization or total hysterectomy. In each case, two pieces of tissue were resected from the pathologic locus, which was defined both visually and colposcopically, and one piece of morphologically normal epithelium (control) was excised from the contralateral side of the cervix outside the pathologic zone. Tissue samples were immediately submerged into RNA-stabilizing solution RNALater (Qiagen) or RPMI-1640 medium (Gibco), then frozen and stored at −80°C. For all patients the original diagnosis was verified by histomorphological examination.

Venous blood sampling was done right before the surgery or any other treatment. The fraction of peripheral blood mononuclear cells (PBMC) was isolated by standard procedure in Ficoll density gradient (Paneco, Russia). Forty-five samples of peripheral blood were also taken from healthy nonpregnant HPV-negative women comparable in age and anamnesis, with no pathology of the cervix (control blood group 1, age characteristics: 23.4 ± 0.9 ($n$ = 15); 33.3 ± 1.7 ($n$ = 15); 46.7 ± 11.1 ($n$ = 15)). For patients with CIN 3 and CC stage IA, blood samples were again collected in 1 and 3 months after conization and course of immunomodulatory therapy. Control group 2 consisted of patients with CIN 3 ($n$ = 15) and CC stage IA ($n$ = 15) (average age 34.1 ± 7.2) who underwent only surgical treatment. Patients of the examined groups did not differ in anamnesis, virological, and histological findings.

Screening for the presence of HPV DNA and identification of HPV genotype were performed by polymerase chain reaction (PCR) using AmpliSens HPV HCR Screen kit (The Central Research Institute of Epidemiology of The Federal Service on Customers' Rights Protection and Human Well-being Surveillance, Russia) and TaqMan probes. E6/E7 oncogene mRNA was detected by the reverse transcription coupled PCR (RT-PCR), and reagents kits were purchased from DNA-Technology and Sileks companies (Russia). The PCR products were visualized by 2% agarose gel electrophoresis. The distribution of HPV genotypes is displayed in Table 1.

| | HPV16 | HPV16, 18, 31, 33 | HPV18 | HPV31 | HPV33 |
|---|---|---|---|---|---|
| CIN 3 | 52.1 | 34.8 | 13.1 | - | - |
| CC stage IA | 79.4 | - | 11.8 | 5.9 | 2.9 |
| CIN 3 + CC stage IA | 61.2 | 23.3 | 12.6 | 1.9 | 1.0 |
| CIN 3 + CC stage IA (3 months after treatment) | 1.1 | 2.2 | 1.1 | - | - |

**Table 1.** The distribution (%) of HPV genotypes in groups of patients diagnosed with CIN 3 or CC of stage IA.

### *2.3.2. Real-time PCR*

Total RNA from tumor cells and PBMC was extracted with TRizol reagent (Invitrogen, USA) following the manufacturer's guidelines. The concentration and purity of the RNA template was determined by spectrophotometry (BioWave II+, Biochrom, UK). RNA nativity was

determined by capillary gel electrophoresis using Experion Automated Station and RNA StdSens analysis kit (Bio-Rad, USA). The extracted RNA template was treated with DNase I (Fermentas, ThermoScientific, USA). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using random hexaprimers and ProtoScript MMLV reverse transcriptase following the protocol proposed by the manufacturer (New England BioLabs, UK). RNA and cDNA samples were stored at −80°C. Gene expression was estimated by SYBR Green real-time PCR. Amplification was performed in StepOnePlus thermal cycler (Applied Biosystems, USA) with StepOne™ Software v2.2.2 using 20 ng of cDNA per 1 reaction volume (25 μl) and qPCRmix-HS-SYBR+HighROX 5×-reaction mix (Evrogen, Russia), containing gene-specific primers at final concentration 0.5 μM. Primers for the nucleotide sequences of the investigated genes were selected from published sources (Table 2). Oligonucleotides were synthesized by the Evrogen company (Russia). The PCR protocol was 20 s at 95°C, 20 s at 60°C, and 30 s at 72°C (45 cycles). All the reactions were done in duplicates. Effectiveness of amplification (%) was monitored by standard curve approach. To determine the specificity of primer annealing, the PCR fragments were melted: 1 min at 95°C, 1 min at 60°C, and 10 s at 60°C (80 cycles, the temperature raised by 0.5°C in each cycle). To exclude the possibility of the template cDNA being contaminated by the genomic DNA, PCR was performed for each template under the same conditions with the RNA matrix (negative control). The resultant reaction products were also separated in 8% polyacrylamide gel using the Tris−borate buffer, then stained with 1% ethidium bromide solution and visualized in transmitted UV light (with GelDoc-It Imaging System, UVP, USA). The correspondence of amplicon sizes to theoretically expected ones was confirmed using the low-molecular pUC19/Msp I fragment length marker (Syntol, Russia). Gene mRNA expression was measured using the $2^{-\Delta\Delta Ct}$ method [32]. cDNA samples from normal epithelial tissue or from PBMC of healthy donor were used as calibrator.

| Gene | Sequence | PCR product length | Source |
|---|---|---|---|
| GAPDH F | 5′-GAAGGTGAAGGTCGGAGTC-3′ | 225 | [33] |
| GAPDH R | 5′-GAAGATGGTGATGGGATTTC-3′ | | |
| Caspase 6 F | 5′-ACTGGCTTGTTCAAAGG-3′ | 181 | [34] |
| Caspase 6 R | 5′-CAGCGTGTAAACGGAG-3′ | | |
| Caspase 3 F | 5′-ATGGAAGCGAATCAATGGAC-3′ | 240 | [35] |
| Caspase 3 R | 5′-ATCACGCATCAATTCCACAA-3′ | | |
| Caspase 9 F | 5′-AACAGGCAAGCAGCAAAGTT-3′ | 246 | [35] |
| Caspase 9 R | 5′-CACGGCAGAAGTTCACATTG-3′ | | |

**Table 2.** Primers for the nucleotide sequences of the genes under study.

### *2.3.3. The enzyme activity of caspases*

The enzyme activity of caspases was determined by standard technique using specific substrates labeled with fluorescent marker (7-amino-4-trifluoromethylcumarin—AFC) (Bio-

Rad, USA), detected by variations in fluorescence or optical density [36]. Fifty microliters of lytic buffer prepared by mixing 920 μl of bidistilled $H_2O$, 40 μl of 25-fold reaction buffer, and 10 μl of each of the four inhibitors: phenylmethylsulfonyl fluoride (PMSF) (35 mg/ml), pepstatin A (1 mg/ml), aprotinin (1 mg/ml), and leupeptin (1 mg/ml), were added to the tumor sample (5 mg) or PBMC ($10^6$ cells). The 25-fold reaction buffer included the following components: 250 mM HEPES, pH 7.4, 50 mM EDTA, 2.5% 3-((3-chloramidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), and 125 mM dithiothreitol. After that, the cells were frozen three times in liquid nitrogen, the cell lysate then centrifuged in a microcentrifuge at 17,000*g* (4°C) for 30 min, and the supernatant (template) collected. The activity of caspases 3, 6, and 9 was determined in the reaction buffer by mixing the template with the corresponding specific substrate. The substrate for caspase 3 was DEVD (Asp–Glu–Val–Asp), for caspase 6—VEID (Val–Glu–Ile–Asp), for caspase 8—LETD (Leu–Glu–Thr–Asp), and for caspase 9—LEHD (Leu–Glu–His–Asp). The amount of cleaved AFC was measured by spectrophotometry in FluoroMax ("Horiba-Scientific," Japan) at 395 nm 30, 60, 90, 120, 150, and 180 min after the onset of the reaction. Then, the curve of caspase activity depending on the template and substrate incubation time was plotted. Relative proteolytic activity was calculated as the slope $\Delta S/\Delta t$, where

$$\Delta S = \left[S(t_i) - B(t_i)\right] - \left[S(t_0) - B(t_0)\right],\ \Delta t = (t_i - t_0),$$

where $S$ is the sample signal at time $t$, and $B$ is the blank signal at time $t$, $t_i$ is the time of measurement, and $t_0$ is the time of initial measurement.

*2.3.4. Flow cytometry*

Total leukocyte fraction was prepared via using ammonium chloride osmotic shock (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM $Na_2EDTA$), and 100 μl of whole blood was taken for each probe. Cells were centrifuged and washed with Versene solution (0.02% $Na_2EDTA$ in phosphate-buffered saline). To measure the surface expression of CD antigens, the cells were probed with fluorophore-conjugated monoclonal antibodies (mAbs): CD3-APC, CD4-FITC, CD8-FITC, and CD95-RPE (Dako, Denmark). One hundred microliters of cell suspension was incubated with mAbs (1:20) for 30 min at room temperature. To prevent nonspecific Fc receptor-mediated mAb binding, the FcR Blocking Reagent was used (Miltenyi Biotec, Germany) in accordance with the manufacturer's instructions. Analysis was performed with MACSQuant Analyzer flow cytometer (Miltenyi Biotec.). Figure 2 describes the scheme for discriminating cell subpopulations of interest (the gating strategy); lymphocytes were gated by forward and side scatter. The numbers of lymphocytes having the following phenotypes were evaluated by the fluorescence parameters: $CD3^+$ (T-lymphocytes), $CD3^+CD4^+$ (T-helpers), $CD3^+CD8^+$ (T-killers), $CD3^+CD95^+$, $CD3^+CD95^{high}$, $CD3^+CD4^+CD95^+$, $CD3^+CD8^+CD95^+$, $CD3^+CD4^+CD95^{high}$, and $CD3^+CD8^+CD95^{high}$. Not less than 100,000 cells were analyzed in each probe. Dead cells were excluded from analysis by propidium iodide staining.

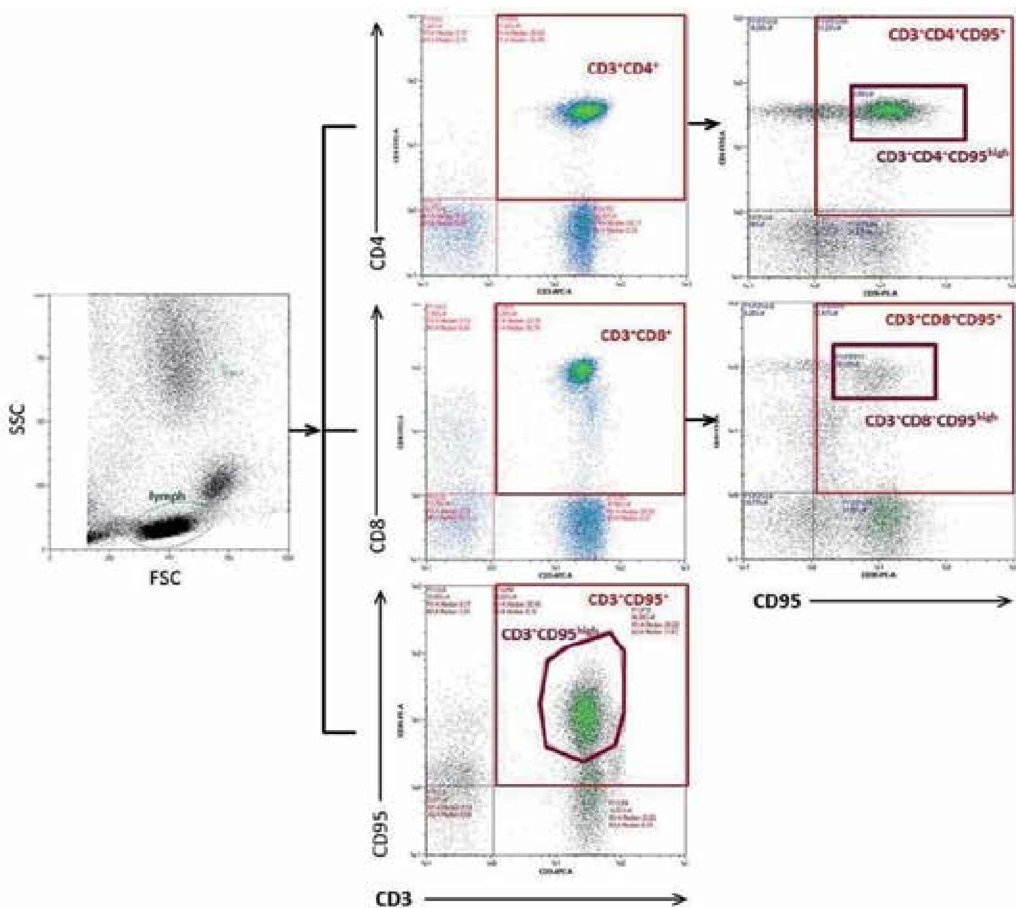


**Figure 2.** The scheme of the flow cytometric analysis of peripheral blood lymphocytes. Statistical reliability of the obtained results was estimated using the Student *t*-test and the nonparametric Wilcoxon–Mann–Whitney test. One-way ANOVA was also performed to compare between control/cancer groups of samples.

## 2.4. Results and discussion

### *2.4.1. Expression and activity of caspases in cervical intraepithelial neoplasia grade 3 and cervical cancer tissue samples*

In the first phase of our research, we assessed the level of the relative mRNA expression of the initiator caspase 9 and executioner caspases 3 and 6 at different steps of CC development, starting from CIN 3 to advanced cancer (stages II–IV) comparing to the normal epithelium. The results are summarized in Table 3. Substantial fraction of CIN 3 samples (more than 50%) displayed the control level of caspases 3, 6, and 9 expressions, while the downregulation of caspase 3 and caspase 9 mRNAs was observed in the one-third of CIN 3 samples; caspase 6 was downregulated in 20% of samples. In the minor portion of CIN 3 cases (less than 20%),

the relative amount of caspase 3, caspase 6, and caspase 9 transcripts was increased compared to the control. Microinvasive cancer (stage IA1) showed elevated mRNA levels for all three caspases in 50% of cases, with other cases displaying no difference or decrease (of various extents) relative to the control level. Analysis of stage II–IV CC samples revealed that mRNA levels of caspases 3, 6, and 9 generally did not differ from those of normal epithelium. For the rest of the advanced cancer samples, the upregulation of caspases 3 and 6 was detected, with caspase 9 showing inverse relation. In summary, cervical cancer progression was not found to be significantly correlated with mRNA expression of caspases 3, 6, and 9.

| | CIN 3 (n = 25) | CC stage IA (n = 12) | CC stage II–IV (n = 10) |
|---|---|---|---|
| **Caspase 3** | Control, 56% (*n* = 14) | Control, 33% (*n* = 4) | Control, 60% (*n* = 6) |
| | ↑ , 16% (*n* = 4) | ↑ , 50% (*n* = 6) | ↑ , 40% (*n* = 4) |
| | ↓ , 28% (*n* = 7) | ↓ , 17% (*n* = 2) | |
| **Caspase 6** | Control, 60% (*n* = 15) | Control, 25% (*n* = 3) | Control, 70% (*n* = 7) |
| | ↑ , 20% (*n* = 5) | ↑ , 50% (*n* = 6) | ↑ , 30% (*n* = 3) |
| | ↓ , 20% (*n* = 5) | ↓ , 25% (*n* = 3) | |
| **Caspase 9** | Control, 52% (*n* = 13) | Control, 42% (*n* = 5) | Control, 40% (*n* = 4) |
| | ↑ , 16% (*n* = 4) | ↑ , 50% (*n* = 6) | ↑ , 60% (*n* = 6) |
| | ↓ , 32% (*n* = 8) | ↓ , 8% (*n* = 1) | |

**Table 3.** The change of mRNA levels of caspases at CIN 3 → CC progression relative to the normal epithelium. Arrows (↑ or ↓ ) correspond to up- or downregulation relative to the control level.

As modulation of caspases proteolytic activity is regarded as the highest level of regulation, we further explored its relative change in normal epithelium, CIN 3, and invasive CC specimens using specific tetrapeptide fluorescently labeled substrates. In 38% of CIN 3 samples tested, caspases 3 and 6 exhibited increased activity compared to the control level, with caspase 9 activity being upregulated only in a few (14%) CIN 3 cases (Figure 3). As for the rest of CIN 3 samples, the protease activity of caspases was found to diminish or correspond to the control level. In those CIN 3 samples that exhibited altered caspase 3 activity, either downregulation or upregulation was significant as compared to the normal epithelium (Figure 3A). In contrast to caspase 3, the activity of caspase 6 matched the range of control values in 50% of CIN 3 cases; the other 50% of tissue samples demonstrating significant (2.5- to 3-fold) increase in caspase 6 activity (Figure 3B). The activity of caspase 9 in CIN 3 group was generally comparable to that of the control group (Figure 3C). Stage IA was characterized by significant decrease of activity of all caspases analyzed (for caspases 3 and 9, the median activity values were significantly lower than those of the control samples); the same trend was observed for stages II–IV of CC. Note that in 23% of CC stage IA samples, the activity of caspase 6 was notably increased.

To summarize, a gradual downregulation of caspases 3, 6, and 9 occurs as invasive CC progresses, while at the stage of preinvasive cancer (CIN 3) the activity of caspases 3 and 6 may be significantly higher than that of normal epithelium. This observation may indicate, in respect of apoptosis-associated processes, a high degree of molecular heterogeneity of lesions morphologically defined as CIN 3. The activity of caspases 3 and 9 was shown to be signifi-

cantly correlated with the clinical stage ($r = -0.72$, $R^2 = 0.52$, $p < 0.01$ for caspase 3; $r = -0.67$, $R^2 = 0.45$, $p < 0.01$ for caspase 9; linear regression). The correlation between caspase 6 activity and CC stage was not proved to be statistically significant. Comparing the results on the expression level of caspases 3, 6, and 9 mRNA, on the one side, and the change of their protease activity, on the other side, it can be inferred that, for the invasive forms of CC, these two regulatory levels (i.e., transcriptional and translational) are poorly correlated: as the activity of caspases decreases, their mRNA levels stay within the range of control values or increase. At the same time, mRNA expression and protease activity values were comparable with respect to CIN 3 lesions.

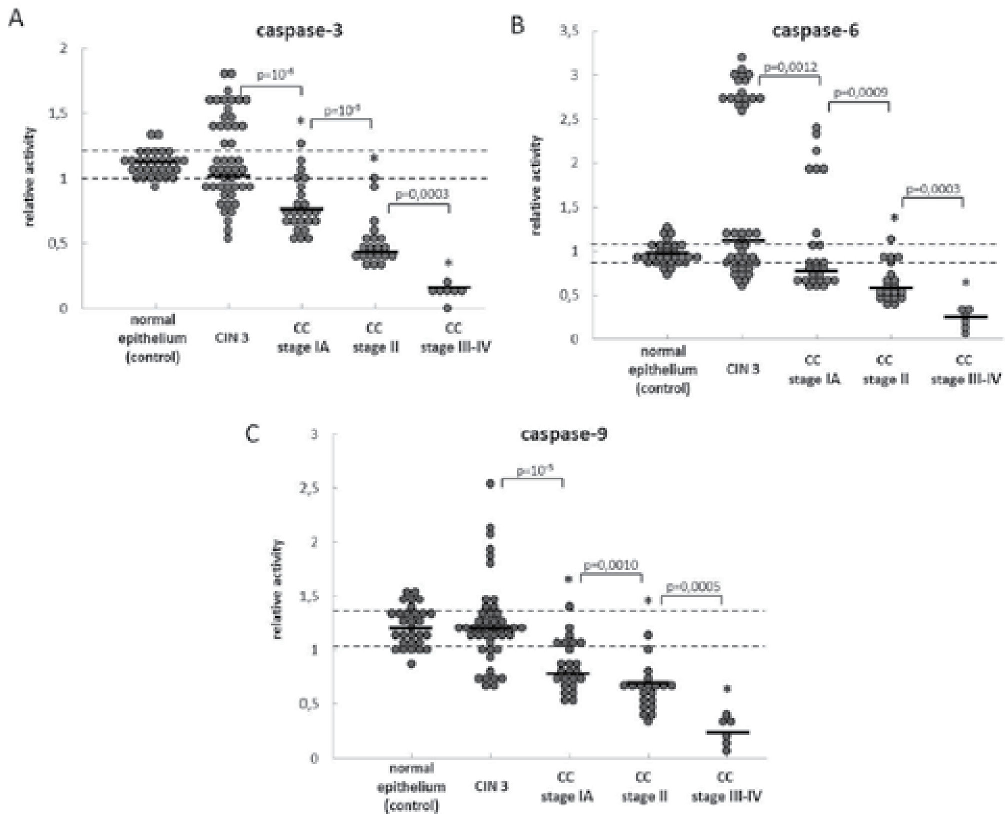


**Figure 3.** Scatter plots showing individual patients' changes of protease activity of caspases 3(A), 6(B), and 9(C) in pathological tissue at different stages of CC development. Horizontal bars represent the median activity level for each data set; dashed lines indicate ± standard deviation (SD) of the mean value in samples of normal epithelium (control). The results were multiplied by $10^4$ for convenience. *Patient groups with median caspase activity being significantly different from that of control (the differences were considered significant if $p$-value was <0.05, $U$-test). The $p$-values shown on the plots were obtained by comparing successive stages of CC.

The above data allow us to speculate that the growth and development of CC is accompanied by multifaceted impairments of apoptotic processes implemented in the form of global suppression of caspases' functioning and multiple resistance of tumor cells to apoptotic stimuli. To our knowledge, this is the first systematic study investigating the changes of

expression/activity of functionally different caspases at different clinical stages of cervical cancer. Caspase 3 is a universal executioner caspase of both "extrinsic" and "intrinsic" apoptotic pathways. Caspase 6 represents one of the least studied members of the family, although new evidence indicates its crucial role in amplification of apoptotic signal; as distinct from other executioner caspases (3 and 7), caspase 6 is capable of being autoactivated. However, its elevated activity does not necessarily result in apoptosis [37]. It was also revealed that the range of caspase 6 targets does not overlap with that of other effector caspases [38]. Caspase 9 is a crucial intracellular sensor of apoptogenic stimuli such as hypoxia, genome destabilization, redox dysregulation, deficiency of prosurvival, and growth factors—all these stimuli inevitably affect the process of tumor development as a consequence of rapid accumulation of cell mass and overexpression of HPV oncogenes. Therefore, the suppression of caspase 9 is required to escape their influence. It is worthy of notice that the inhibition of caspase activity is an early event in CC progression since it was observed in the majority of stage IA1 samples (i.e., invasive microcarcinoma) and apparently reflects the establishment of an aggressive phenotype of CC cells.

The principal conclusion that can be made on the basis of our study and data available from literature is that caspases occupy the key position in the multistep model of natural CC development and have prospects of being used as biomarkers for this type of oncopathology. The importance of studying the profile of expression/protease activity of caspases with the use of cervical tissue samples is emphasized in the work of Arechaga-Ocampo et al. [39], where the authors come to a conclusion that the activity of caspases upon CC development is determined by the individual spectrum of genomic aberrations and the microenvironment. Therefore, the information on caspases expression patterns derived by the use of model cell systems describes isolated cases rather than reflects the *in vivo* situation. In view of this aspect, the elevated levels of activity of caspases 3 and 6 that we observed in CIN 3 samples seem to be of a particular interest. Indeed, there are some published reports arguing for frequent upregulation of apoptotic markers (caspases, internucleosomal DNA fragmentation) in clinical tissue specimens of CIN 3 [40–44], but nevertheless, the data pointing at the absence of such alterations should also be taken into consideration [45]. At this point, it is difficult to judge objectively whether the increase of caspase activity is linked to the higher risk of a subsequent fast progression of CIN to invasive cancer or, in contrast, the probability of long-term disease persistence without signs of invasive growth. To understand the biological significance of certain changes in activity of caspases and the driving forces underlying these changes upon CC development, it is necessary to continue research with inclusion of other regulatory molecular factors. Caspases as being multifunctional proteinases may participate in some processes that rule the HPV life cycle progression [46]. In particular, there is evidence showing that production of E-proteins is coupled with the increase of stability and activity level of caspases [47]. It was also discovered that HPV oncoproteins contain specific sites for caspase-dependent proteolysis [48]. The study of Moody et al. [46], carried on with the use of organotypic raft model of stratified epithelium, demonstrates the potential role for the proteolytic activity of caspases in the HPV life cycle. According to Moody et al., in raft cultures generated from normal keratinocytes, the active (processed) form of caspase 3 was present in small amounts and was concentrated predominantly in the basal cell layer. In similar cultures

composed of HPV31-infected keratinocytes, the active form of caspase 3 could be detected in all layers of epithelium; however, these two model cell systems revealed no difference in the amount of inactive caspase 3 (procaspase 3). Taken together, these data seem to be in poor agreement with the idea that the main influence of HPV oncoproteins is directed to the promotion of S-phase and inhibition of apoptosis. However, as argued by the authors [46], the high content of the activated form of caspase 3 observed in HPV-positive raft cultures was not coupled with any of the morphological features of apoptotic death. Moody and coauthors [46] proved that in HPV-positive cells, the activity of caspases, although being elevated, could not reach a "barrier" value sufficient to switch on the program of apoptosis. The HPV proteins presumably provide high threshold of sensitivity to the caspase-mediated reactions through potentiating the expression of antiapoptotic proteins—Bcl-2, survivin, IAPs, as well as other factors. The intricacy of mechanisms of shifting the balance between pro- and antiapoptotic regulators becomes even more evident in the light of new facts about apoptogenic effect of the E2F transcription factor: as it was mentioned earlier, hyperexpression of E7-oncogene liberates E2F, whose targets, along with proliferative genes, comprise genes encoding proapoptotic proteins Puma, Noxa, and Bim [4].

Indeed, in recent years, researchers pay much attention to the expression of endogenic inhibitors of caspase activity in cervical neoplasia and cervical cancer in view of the problem of multiple resistance of CC to programmed death. Such inhibitors are, for example, the members of IAP-family—XIAP and survivin. XIAP is known for its ability to bind caspases 3, 8, and 9, thereby preventing their activation, and, owing to its ubiquitin-ligase activity, to target proapoptotic factors for proteasomal degradation. The upregulation of XIAP has been documented in CIN and CC samples [49]; the involvement of XIAP in the maintenance of CC resistance to cisplatin and doxorubicin has been discussed as well [50]. Similarly, the disease progression occurred to be associated with upregulation of survivin that is able to inactivate caspases 3, 7, and 9 via direct binding to them [29, 51, 52]. The study of Lu et al. [53] revealed the negative correlation between survivin and caspase 3 expression levels in CC specimens by immunohistochemistry. In the work performed by Cao et al. [51], it is proposed to consider the survivin expression level as a marker enabling evaluation of the risk of CIN progression to invasive cancer. As is reported by Espinosa et al. [54], it is survivin that becomes overexpressed with CC stage progression, while other IAP members generally exhibit reduced expression in order to compensate survivin levels (the existence of such a compensatory relationship between the IAP-family members was established by experimental cell systems). Measuring survivin and caspase 3 expressions in both primary tumors and CC metastatic loci allows the prediction of a patient's response to chemotherapy (used in adjuvant or neoadjuvant settings) [53, 55, 56]. Closely, homologues to survivin is the recently discovered IAPs member —a livin protein [57] that was shown, similar to survivin, to be upregulated in cervical neoplasms and revealed to be negatively correlated with the level of caspase 3 expression [58]. There are some other inhibitors of the caspase-mediated pathway known to function by mechanisms distinct from IAPs, as for example c-FLIP. Interacting with FADD, c-FLIP prevents from caspase 8 recruitment and interferes with DISC formation upon activation of death receptor; an apoptosis inhibitory complex (AIC) is formed instead, thus prohibiting initiation of the caspase cascade [59]. The upregulation of c-FLIP in CIN and CC biopsies was also detected by several research groups [60, 61]. The role of c-FLIP in the development of CC

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polyresistance to the wide range of proapoptotic agents is being investigated in parallel with the search of approaches to abrogate its function; for instance, it has been recently discovered that histone-deacetylase inhibitors can induce transcriptional repression of c-FLIP gene thus governing sensitization of CC cells to apoptosis [62]. In contrast to the protein inhibitors of apoptosis mentioned above, endogenic antagonists of IAPs (as for example, Smac/DIABLO) are poorly studied in CC, although their intracellular amounts may appear as important for determining the degree of CC cells' resistance/sensitivity to apoptosis as that of IAPs.

Thus, the reasons and the mechanisms that might account for the observed changes of the caspase activity pattern in the course of CC development appear to be quite diverse. The need to decipher these regulatory interrelationships is determined by the search for solutions to restore the functionality of apoptotic cascade, to increase the effectiveness of chemotherapeutic agents, and to overcome multidrug resistance of CC cells. For example, AT-406, a low molecular weight compound that imitates the binding of Smac/DIABLO to XIAP, can thus block the function of inhibitors of apoptosis, that is why the use of AT-406 or other Smac mimetics provides an opportunity to reactivate caspase 9 [63]. Altogether, the above-stated data highlight the importance and topicality of further careful investigation of caspases and endogenous factors governing their activity in the context of the problem of cervical cancer diagnostics and treatment.

*2.4.2. Expression and activity of caspases and CD95 level in PBMC from patients with CIN 3 and CC*

The expression of either HPV-specific or tumor-associated antigens by neoplastic CC cells and exposure of these antigens to the immune system eventually drive to the activation of specific lymphocyte subpopulations. At the same time, the activation of immunocompetent cells can proceed in parallel with induction of apoptosis that may predetermine, at least partially, the inefficacy of antiviral/antitumor reactions of the immune system. Although there is some published data showing the upregulation of apoptosis in lymphocytes infiltrating cervical cancer tissue [27, 28], the following questions remain under investigation: To what extent apoptosis-associated processes are present in systemic circulation of CC patients? What signaling pathways might be involved in this case? Could the apoptosis-related changes be detected in peripheral blood of patients with preinvasive cancer? Within the task of testing assumption that the mechanisms governing the apoptotic program do exacerbate in the circulating lymphocytes upon CC development, especially with regard to its early stages, we analyzed the level of protease activity of receptor-regulated caspase 8, executioner caspases 3 and 6, and caspase 9 initiating the intrinsic apoptotic pathway, in the mononuclear fraction of blood samples, taken from women with CIN 3 or CC, in comparison with the group of healthy controls (Figure 4).

In 50% of CIN 3 cases, the activity of caspase 8 matched the control level, whereas in the rest 50%, it was notably increased. In invasive cancer, the activity of caspase 8 was significantly higher (in 100% of samples) than the control level, showing strong positive correlation with the stage of the disease ($r = 0.92$, $R^2 = 0.86$, $p < 0.01$) (Figure 4A). Similar correlation was observed for caspase 6: in 100% of invasive CC patients, its activity was significantly (4–5 times) higher relative to the control group ($r = 0.77$, $R^2 = 0.59$, $p < 0.01$); however, unlike caspase 8, in most of CIN 3 cases (77%), the activity of caspase 6 corresponded to the control values (Figure 4B). The relationship between the level of caspase 3 activity and the stage of cancer occurred to be

nonlinear: upon CIN 3 → stage IA progression, the caspase 3 activity increased, while with stage II → IV progression, it gradually diminished up to the control levels, in 5 cases falling to almost undetectable levels ($R^2$ = 0.45, $p < 0.01$, polynomial regression of II order). It is noteworthy that for all examined patient groups, there was a certain percent of samples (35% of CIN 3, 30% of CC stage IA, 38% of CC stage II), with caspase 3 activity showing no difference as compared to the control (Figure 4C). In contrast to caspases 3, 6, and 8, caspase 9 exhibited reduced activity as the stage progressed ($r = -0.60$, $R^2 = 0.36$, $p < 0.01$); however, in 26% of stage IA blood samples, elevated caspase 9 activity was detected. In CIN 3 group, reduced caspase 9 activity was revealed for 40% of samples (Figure 4D). We also examined whether these caspase-specific changes observed at the level of enzymatic activity could extend to the transcriptional level. The correlation of the relative mRNA expression levels of caspases 3 and 6 with the cancer stage displayed, in general, the same character as the activity level, being, however, much less pronounced (data not shown). As for caspase 9 mRNA level, it was not found to be correlated with the stage of CC.

Summarizing the findings stated above, we can conclude that CC progression is associated with the specific change of activity pattern of caspases 8, 3, and 6 that are united by being components of extrinsic, receptor-mediated pathway of apoptosis. Importantly, the systemic fluctuations of caspase activity revealed in the circulating PBMC appear to be an early event in CC development—upregulation of all three caspases were already detectable at the stage of microinvasion for the most of samples, and for substantial portion of samples—at the stage of intraepithelial cancer (CIN 3). It is obvious that molecular factors and mechanisms by which HPV or a developing neoplasia can exert systemic influence on the immune system still remain largely unknown and define trends of future research, but nevertheless there is growing body of evidence that the development of a malignant process can raise considerable changes in gene expression profile of peripheral blood leukocytes, with certain fraction of genes being related to apoptosis signal transduction and implementation of the cell suicide program [64–66]. The upregulation of caspases 8, 3, and 6 activity observed in our study may represent a direct consequence of increased membrane expression of cell death receptors—CD95/APO-1/Fas first of all as the key acceptor of apoptotic signals on the surface of lymphocytes. An increase in the number of CD95-expressing peripheral blood lymphocytes was revealed for patients with hepatocarcinoma [67], melanoma [68], ovarian cancer [69], head and neck cancer [70], gastric [71], nonsmall cell lung cancer [72]. That is why we decided to examine if a similar phenotypic change of circulating lymphocytes could occur along with CC progression.

By using flow cytometric assay, we explored the level of surface expression of CD95-marker in the blood lymphocytes of CIN 3 and CC patients in comparison with the control group. As follows from Figure 5, the number of CD95-expressing cells in CIN 3 group was higher than that of control, exhibiting further increase with CC stage progression. Together with our data on activity of caspases, these results allow us to assume that circulating lymphocytes become more susceptible to Fas-mediated apoptosis. It is worth mentioning, however, that CD95 represents a marker with "dual" functionality: performing a function of a cell death receptor, CD95 serves at the same time as an early activation marker of T-lymphocytes [73]. Taking into account the fact that cervical neoplastic lesions develop on the ground of chronic HPV infection, one can connect the observed elevation of CD95 to the processes of activation of the T cell-mediated branch of immunity, induced by the sustained expression of viral antigens

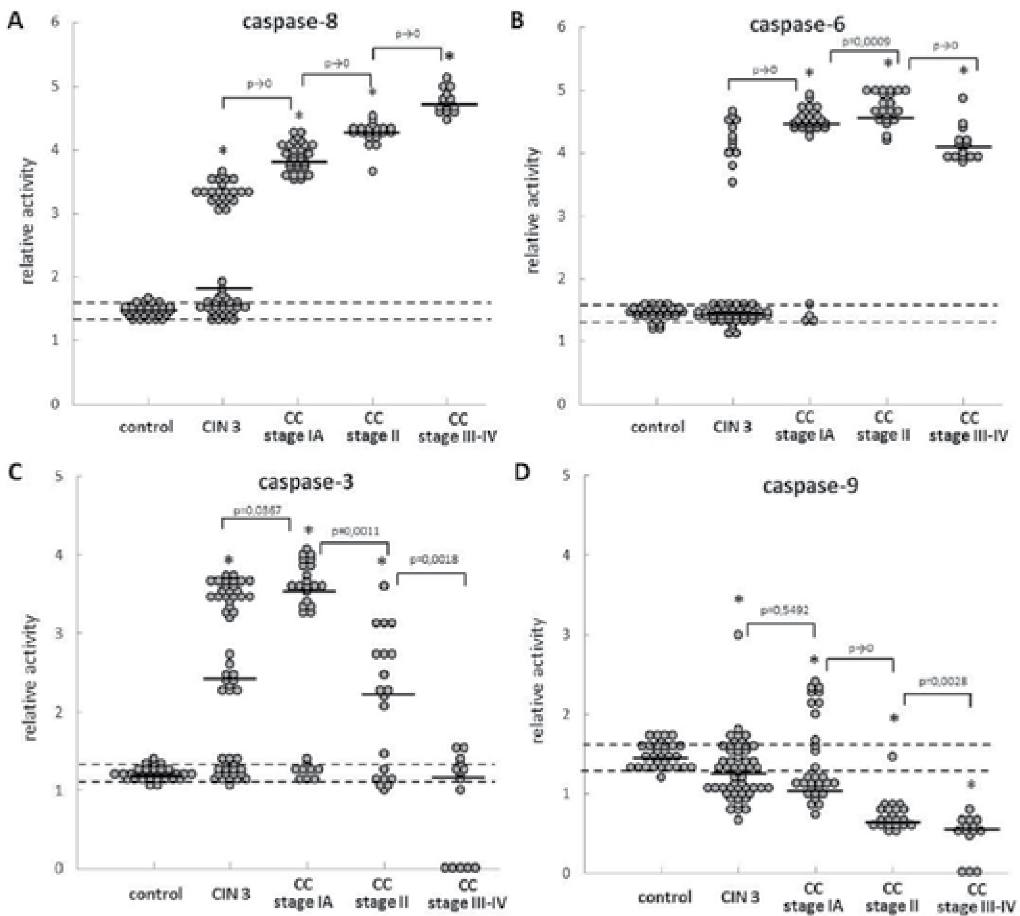


**Figure 4.** Scatter plots showing individual patients' changes of protease activity of caspases 3(A), 6(B), 8(C), and 9 (D) in PBMC at different stages of CC development. Horizontal bars represent the median activity level for each data set; dashed lines indicate ± standard deviation (SD) of the mean value in samples of the control group. The results were multiplied by $10^4$ for convenience. *Patient groups with median caspase activity being significantly different from that of control ($p < 0.05$). The *p*-values shown on the plots were obtained by comparing successive stages of CC.

and their exposure to the antigen-presenting cells. Considering this potential cause, we narrowed the analyzed population by adding a pan T-cell marker (CD3) and determined the number of lymphocytes having $CD3^+CD95^+$ or $CD3^+CD95^{high}$ phenotype in the blood of CIN 3 patients, as compared with the control group (Figure 5). A significant increase in the percentage of $CD3^+CD95^{+/high}$ T-cells regarded as effector T-lymphocytes was found in CIN 3 women. Furthermore, we have seen that this change of CD95 expression affected primarily the subpopulation of T-helpers ($CD3^+CD4^+CD95^+$ and $CD3^+CD4^+CD95^{high}$ phenotypes) (Figure 5) but was not characteristic of cytotoxic T-cells (defined by $CD3^+CD8^+CD95^{+/high}$ phenotype), thus supporting the results of other studies disclosing the dominant role of the T-helper branch of immunity in the processes of CC carcinogenesis (reviewed in [74, 75]).

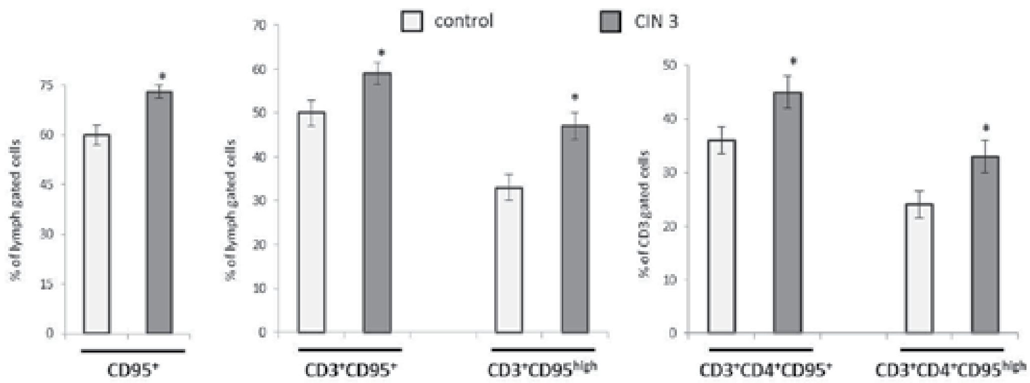


**Figure 5.** Expression of CD markers in peripheral blood lymphocytes of CIN 3 patients. **p* < 0.05 (*U*-test).

Does the intensification of the apoptotic death of circulating leukocytes take place in the blood of women suffering from CIN or cervical cancer, and, if so, under the influence of what humoral factors does it occur? Which of the cell subpopulations becomes the most susceptible one? What is the impact of apoptotic processes on immune status and disease progression? To answer these questions, further research is undoubtedly needed to be done. Of special interest are those immune state disorders that support the persistence of HPV infection and promote transition of the disease from intraepithelial form into true cancer and microcarcinoma. At the current step of our research, additional corroboration of potentiation of apoptosis-related reactions in the blood lymphocytes upon CC development comes from observation that in the course of treatment, the immune status recovery process was accompanied by normalization of the level of CD95-marker expression and activity of caspases 8, 3, and 6.

*2.4.3. Activity of caspases and CD95 level in PBMC of patients with CIN 3 and CC after treatment*

At present, new approaches to immunotherapy/immunomodulatory therapy for the HPV infection and cervical neoplasms designed for the reduction of the HPV clearance period, prevention of reinfection, and activation of mechanisms of nonspecific immune defense are being intensively developed (for a review, see, for example [76, 77]). However, the problem of putting such approaches into practice is closely related to the task of finding adequate criteria for assessment of their clinical effectiveness. Besides, regardless of treatment strategy (standard, surgical, or combined, including immunomodulatory therapy), there exists a need to monitor the recovery of a patient's immune status during the postoperative period and to estimate the individual response to therapy, which, in turn, requires an appropriate set of biomarkers. Considering these points, we assessed the change of caspase activity level and CD95 expression in circulating PBMC of women diagnosed with CIN 3 or microinvasive CC (stage IA1) after treatment. Repeated blood samplings were done in 1 and 3 months after diathermoconization and the course of immunomodulatory therapy. The control group 2 was consisted of patients with CIN 3 ($n$ = 15) and CC of IA stage ($n$ = 15), who had received only surgical treatment in full accordance with the approved standard. As it follows from Figure 6, in the blood lymphocyte fraction of patients constituting the control group 2, the expression

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of CD95 marker did not alter significantly within the 3-month period, whereas immunomodulatory treatment elicited marked reduction in the number of CD95-positive cells. It is worth to mention that the more noticeable changes of CD95 expression in the group of receiving complex treatment were associated with the more effective elimination of HPV infection.

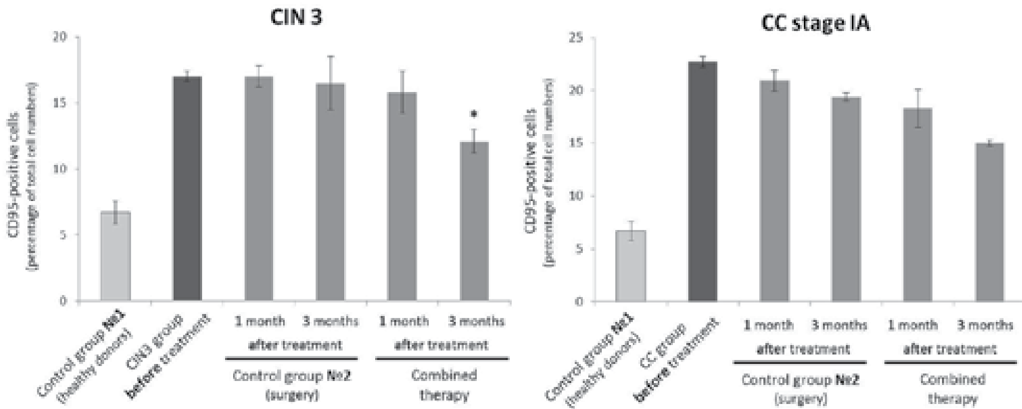


**Figure 6.** Change of CD95 expression in peripheral blood lymphocytes of patients diagnosed with CIN 3 or microcarcinoma of the cervix after treatment. Mean values ± SD are shown. *Significant difference between the control group 2 (3 months after treatment) vs. CIN3 group of patients received combined therapy ($p < 0.05$, *U*-test).

Resting on the obtained results, indicating gradual normalization of the numbers of circulating CD95-positive lymphocytes after the course of treatment, we decided to explore if this change could be coupled with normalization of the activity/expression levels of caspases. After treatment, the change of caspase 8 activity was most pronounced (Figure 7A): in CIN 3 and CC samples, it appeared to return to the control levels after 1 or 3 months, respectively. Only 15% of CIN 3 samples still exhibited the increased activity of caspase 8 after treatment, as compared with 50% before treatment. The activity of caspase 6 (Figure 7B), as opposed to caspase 8, was elevated in 60% of the CIN 3 cases after treatment (although before treatment it was upregulated only in 23% of cases). In CC stage IA group after treatment, caspase 6 activity was revealed to correspond to the control levels for the most of samples tested; however, elevated activity was also detected in several cases. The pattern of caspase 3 activity (Figure 7C) measured in PBMC of patients with CIN 3 and CC stage IA after the treatment course remained actually unchanged, with a slight (statistically nonsignificant) decrease in the median values in each group. In CIN 3 group, both before and after treatment, increased caspase 3 activity levels were observed in 60% and 45% of PBMC samples, respectively. As was stated before, in contrast to caspases 8, 3, and 6, the activity of caspase 9 was shown to diminish with the disease progression (including CIN 3 and CC stage IA). After treatment, caspase 9 activity was shown to match the control level or to be increased (Figure 7D). Interestingly, if before treatment there were no CIN 3 cases with elevated caspase 9 activity (except for a single case), 7 of PBMC samples demonstrated caspase 9 upregulation after the course of treatment. Although it was the level of proteolytic activity of caspases, but not mRNA levels, that displayed the most significant change in CIN 3 and CC stage IA samples, we

nevertheless have analyzed mRNA expression of caspases in PBMC samples of these patient groups after treatment but did not find any significant differences (data not shown).

Altogether, the results obtained indicate that after the course of treatment, a restoration of caspase activity level up to the control values does occur in the blood lymphocytes of CIN/CC patients, with the maximum effect observed for the initiator caspases 8 and 9. By this, we confirm the specificity of changes of the selected indicators, namely, the activity profile of the initiator and executioner caspases, establish their relevance to neoplastic progression, and reinforce the possibility of using caspases as biomarkers of the early steps of cervical cancer development.

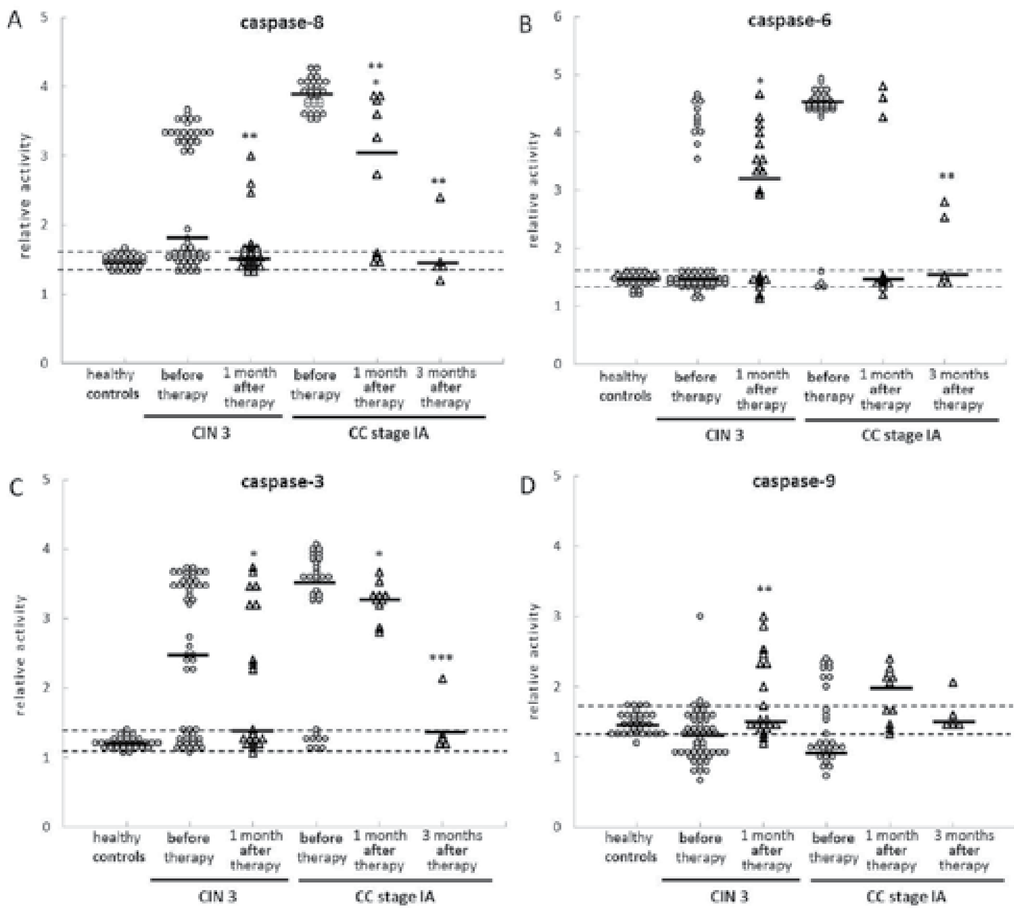


**Figure 7.** Scatter plots showing individual patients' changes of protease activity of caspases 8 (A), 6 (B), 3 (C), and 9 (D) in PBMC of CIN 3 and CC stage IA groups as measured before and after treatment. Horizontal bars represent the median activity level for each data set; dashed lines indicate ± standard deviation (SD) of the mean value in samples of the control group. The differences were considered significant if $p$-value was <0.01 ($U$-test). *Significant difference between the patients and the healthy controls. **Significant difference between caspase activity levels measured before vs. after treatment within the same group. ***Significant difference between 1- and 3-month periods in a group of patients diagnosed with stage IA.

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## 3. Conclusions

By the present study, we intended to show that caspases are pivotal components of the molecular "portrait" of CIN and cervical cancer characterizing the processes of establishing a basic phenotypic trait of a tumor—resistance to apoptosis-inducing factors. By measuring the activity of caspases, we were able to differentiate the early steps of CC progression (intraepithelial and microinvasive cancer) and reveal systemic deviations in the cellular branch of immunity. We suppose that the development of the ability to suppress apoptotic death evaluated by the level of caspase activity can serve as a pointer to malignant transformation of CIN, to the readiness of epithelial cells to counteract the immune reactions, and may predispose to further disease progression. The data accumulated to date provide grounds for considering caspases as promising biomarkers for comprehensive diagnostics of CIN and early forms of CC (in combination with other molecular markers of the viral or cellular origin, as well as with morphological criteria). Nevertheless, it should be noted that more research is required for successful translation of the described markers into clinical practice.

In addition to the prospects of clinical application, the results obtained define possible directions of further fundamental research as, for example, the investigation of molecular factors and mechanisms controlling induction of apoptosis-related processes in immune cells of HPV/CIN-positive individuals, as well as the mechanisms regulating caspase activity in neoplastic cells of the cervix, the elucidation of the impact of apoptotic pathways on systemic immune suppression upon the development of oncopathology. No doubt that the model of regulatory relationships that provide the abrogation of apoptosis in CC cells or its induction in lymphocytes still contain a lot of "gaps" and that is why a great many of the current studies are aimed at identification of the novel molecular players of the apoptotic signaling pathway functionally linked to caspases. For example, it emerges from these studies that TWEAK/Fn14- and TRAIL-mediated pathways of triggering cell death are deeply integrated into the mechanisms of CC development [4, 78]. At the same time, more and more attention is being given to the exploration of alternative variants of PCD and their cross-talk with the mechanism of caspase-dependent apoptosis, for example, there is some new data giving insight into the role of autophagy and endoplasmic reticulum (ER) stress-induced cell death in CC carcinogenesis [79]. Caspases are supposed to function as molecular "switches" between different pathways of programmed death—autophagy and apoptosis—under the influence of different types of treatment on cervical cancer cells [80–82]. These facts emphasize one more time the universality of caspases and stress their importance for studying various aspects of the problem of cervical cancer development and treatment.

## 4. Appendices and nomenclatures

CC—cervical cancer, CIN—cervical intraepithelial neoplasia, DISC—death-inducing signaling complex, HPV—human papillomavirus, IAPs—inhibitors of apoptosis protein family, PBMC—peripheral blood mononuclear cells, PCD—programmed cell death.

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Chapter 14

# The Apoptotic Microtubule Network During the Execution Phase of Apoptosis

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Additional information is available at the end of the chapter

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**Abstract**

Apoptosis is a regulated energy-dependent process of cell death characterized by specific morphological and biochemical features in which caspase activation has a central role. During apoptosis, cells undergo characteristic morphological rearrangements in which the cytoskeleton participates actively. From a historical point of view, this reorganization has been assigned mainly to actinomyosin ring contraction with microtubule and intermediate filaments, both reported to be depolymerized at early stages of apoptosis. However, recent results have shown that the microtubule cytoskeleton is reformed during the execution phase of apoptosis, forming an apoptotic microtubule network (AMN). AMN is closely associated with the plasma membrane, forming a cortical ring or cellular "cocoon." Apoptotic microtubules' reorganization has been reported in many cell types and under many apoptotic inducers. Recently, it has been proposed that AMN is essential for preserving plasma membrane permeability and cell morphology during the execution phase of apoptosis. Apoptotic microtubules' depolymerization leads cells to secondary necrosis and the release of toxic intracellular contents that can harm surrounding cells and initiate inflammation. Therefore, microtubules' reorganization in physiological apoptosis during development and in the adult organism or in pathological apoptosis induced by anticancer treatments or chronic inflammation is essential for tissue homeostasis, preventing cell damage and inflammation.

**Keywords:** apoptosis, microtubules, cytoskeleton

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# 1. Introduction

## 1.1. Apoptosis

Apoptosis is an intracellular signaling pathway conserved across evolution dependent on a caspase-mediated proteolytic cascade that leads to programmed cell death through a series of cellular changes distinct of cell necrosis. Apoptosis plays a critical role in tissue remodeling during development, tissue homeostasis, cleaning of senescent cells, and removal of the cells with severe DNA damage. Given that cell necrosis causes the release of toxic molecules and causes inflammation, an important function of apoptosis is to isolate specific cells and prepare them for disposal by phagocytosis. This program of cell death is carried out by organelle-directed regulators including the Bcl-2 proteins and ultimately executed by proteases of the caspase family.

The apoptotic process can be divided into three functionally distinct phases : (a) induction – cellular environmental changes that result in the activation of intracellular apoptotic mechanisms (entry into the execution phase); (b) execution – processes that result in the degradation of intracellular components by a family of proteases called caspases which cleave a variety of important structural and regulatory proteins at conserved aspartic acid residues to alter their function irreversibly; and (c) cleaning – those events associated with the removal of apoptotic cells and cellular debris by "professional" phagocytes such as macrophages, immature dendritic cells, and neutrophils.

# 2. Apoptosis and secondary necrosis

In contrast to apoptotic cells *in vivo*, cells that perform apoptosis *in vitro* cell cultures are not removed by phagocytes and suffer a late process of secondary necrosis, which is defined as the loss of membrane integrity and release of cell contents into the extracellular space. Secondary necrosis *in vivo* will take place in the presence of massive apoptosis that overloads the ability of available macrophages as well as deficiencies in the number of macrophages and/or saturation of the immune system as seen in chronic inflammation. Secondary necrosis is the natural result of apoptosis in unicellular eukaryotes but can also occur in multicellular organisms and is involved in the genesis of many human pathologies.

Elimination of apoptotic cells by phagocytosis instead of progressing to necrosis secondary has major advantages to multicellular organisms. These advantages include increased speed for cell elimination, increased degradation of cellular component and a most cell-energy-efficient reuse of the components of engulfed apoptotic cells, allowing proper recycling of molecules. Furthermore, and most significantly, apoptotic cell elimination by phagocytosis when the apoptotic cell is still wrapped by a plasma membrane integrated allows that cell disintegration including membrane permeabilization takes place in the secure compartment of the macrophage phagolysosome. This mechanism prevents secondary necrosis, which is potentially pathological through the release of partially degraded cellular components such

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as "damage-associated molecular patterns" (DAMPs) that are pro-inflammatory and immunogenic. Among DAMPs are proteases, nucleosomes, proteolytically processed autoantigens, calcium-binding protein (calgranulin), high mobility group box 1 (HMGB-1), and urate crystals. The cells in secondary necrosis may also be phagocytosed by macrophages *in vivo* following different mechanisms that have been reviewed recently. The removal process of apoptotic cells and cells undergoing secondary necrosis are not identical and generally they have several consequences in terms of the inflammatory and immunogenic responses. Apoptotic clearance *in vivo* includes the sensing of corpses via "find me" signals, the recognition of corpses via "eat me" signals and their cognate receptors, the signaling pathways that regulate cytoskeletal rearrangement necessary for engulfment and the responses of the phagocytes.

While typically the removal of apoptotic cells is anti-inflammatory and "immunologically silent", phagocytosis of cells in secondary necrosis is pro-inflammatory and immunogenic and thus represents another mechanism by which secondary necrosis generates pathogenic consequences. Secondary necrosis, therefore, can produce acute and chronic diseases and it has been recently implicated in a growing number of clinical situations that occur with acute and chronic inflammation including many autoimmune alterations, ischemia, atherosclerosis, chronic obstructive pulmonary disease (COPD), pulmonary inflammation associated with oxidative stress in smokers, cystic fibrosis, asthma, bronquioestasis, and infections. On the other hand, secondary necrosis that affects tumors has recently gained prominence because of its recognition as a process with beneficial implications in anticancer therapies by promoting the activation of the immune system response and consequently the disposal of tumor cells. Chemotherapy and radiotherapy generally act by the induction of apoptosis in tumor cells. It has been demonstrated that after treatment with ionizing radiation or chemotherapeutic agents, cancer cells became highly immunogenic when administrated into immunocompetent mice. Several observations suggest that such immunogenicity is associated with progression of apoptotic cells to secondary necrosis and release of DAMPs molecules.

## 3. Reorganization of the cytoskeleton during apoptosis

The execution phase of apoptosis lasts approximately one hour and is characterized by typical morphological features: cell shrinkage, plasma membrane "blebbing," chromatin condensation, and DNA and cells fragmentation. To perform these dramatic morphological changes that accompany the execution phase of apoptosis, apoptotic cells make a series of profound changes (breaks and rearrangements) in the cell cytoskeleton.

The cytoskeleton is made up of three main types of filamentous proteins, actin filaments, intermediate filaments, and microtubules, that assemble into higher-order polymers in healthy cells and coordinately act to increase tensile strength, allow cell motility, maintain cell morphology, participate in cell division, and provide platforms for positioning and transport of cellular components.

Previous experiments have shown that actinomyosin cytoskeleton plays an essential role in cellular remodeling during the early events of the execution phase of apoptosis while micro-

tubules and intermediate filaments are disorganized. However, some researchers have demonstrated the reorganization of microtubules in apoptotic cells at late stages. This rearrangement occurs during the execution phase of apoptosis, playing a key role in maintaining the integrity of the plasma membrane and dispersion of cellular and nuclear fragments.

Basically, cytoskeletal rearrangements during apoptosis can be summarized as in Figure 1. Initially, actinomyosin ring contraction is activated via phosphorylation of myosin light chain (MLC) II. MLC phosphorylation is under the control of the Myosin Light Chain kinase (MLCK), ROCK (Rho-associated coiled-coil-forming protein) kinases and is regulated by MLC phosphatase (MLCP). It has been discovered that the ROCK kinases actively phosphorylate a large cohort of actin-binding proteins and intermediate filament proteins to modulate their functions. ROCK kinases activates MLC by direct phosphorylation and by inhibiting the activity of MLCP. This movement of the actinomyosin contractile ring is facilitated by the early disruption of microtubules and intermediate filaments, which allows full contraction of the ring and the formation of protrusions of plasma membrane ("blebs"). Subsequently, actin cytoskeleton is depolymerized and, coinciding with the absence of an organized structure of the various elements of the cell cytoskeleton, apoptotic microtubules are repolymerized close to the cytosolic side of the plasma membrane.

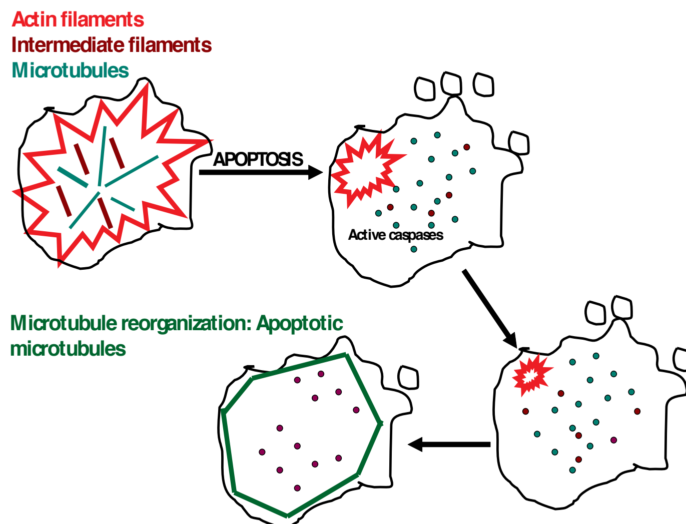


**Figure 1.** Reorganization of the cytoskeleton during apoptosis.

During the execution phase of apoptosis, ROCK I is cleaved by caspase-3 at a conserved DETD1113/G sequence and its carboxy-terminal inhibitory domain is removed, resulting in deregulated and constitutive kinase activity that is necessary and sufficient for actinomyosin ring contraction.

MLC phosphorylation is also regulated by the calcium-dependent MLCK calmodulin. Thus, an increase in calcium may also activate actinomyosin ring contraction. In the ring contraction

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activated by ROCK kinases not only MLCK is involved but also LIM kinase through phosphorylation and inactivation of cofilin, which stabilizes actin polymers.

After actinomyosin ring contraction, actin filaments are depolymerized by the action of active caspases. The Rho effector protein kinase C-related kinase (PRK) 1 is cut by active caspases leading to a constitutively active kinase fragment and after that, PRK1 can induce depolymerization of actin filaments. Likewise, the kinase p21-activated kinase (PAK) 2, a Rac effector, can be activated by caspases and active PAK has been demonstrated to induce stress fibers disassembly. Furthermore, caspases can cleave gelsolin which depolymerizes the actin cytoskeleton in a $Ca^{2+}$-independent manner.

Intermediate filaments that help maintain the integrity of tissues and cells are disrupted at the onset of apoptosis by the action of caspases. The intermediate filament cleavage causes fragmentation and aggregation and the breaking of the nuclear lamins facilitates nuclear disintegration. Cleavage occurs at a conserved location within the rod domain, causing loss of filament integrity and disorganization of the nuclear and cytoplasmic intermediate filaments networks. Caspase cleavage of intermediate filaments is important for the timely execution of apoptosis as evidenced by the delay incurred when caspase-insensitive forms of lamins and desmin are overexpressed. Equally interesting, the caspase 6-mediated cleavage of lamin A/C is required for complete chromatin condensation during apoptosis. The early onset and efficient cleavage of intermediate filaments proteins may be fostered by physical proximity as key effectors of the apoptotic machinery, including procaspase 3, bind cytoplasmic intermediate filaments.

After microtubules, actin, and intermediate filaments' depolymerization, apoptotic cells are devoid of the structured elements of the cytoskeleton. Then, microtubules are reorganized leading to the formation of the apoptotic microtubule network (AMN), which becomes the only element of the cytoskeleton during the execution phase of apoptosis (Figure 2).

The molecular mechanism involved in the early microtubule depolymerization during the execution phase of apoptosis is unknown although several hypotheses have been postulated. Microtubule dynamics is governed by several effectors, microtubule-associated proteins (MAPs), motor proteins such as kinesin, gradients Ran-GTP+ends proteins and proteins that bind to tubulin. These microtubule-associated proteins are in turn under the control of phosphatases and kinases. One of these regulatory kinases CDK1 is associated with cyclin B, a key enzyme for the entry into mitosis and essential for mitotic spindle formation. One of the activities ascribed to CDK1 is the interphase microtubule depolymerization at the beginning of mitosis. Among CDK1 substrates are numerous microtubular effectors such as MAP4 and XMAP215 that when are phosphorylated their ability to stabilize microtubules may be reduced. Furthermore, CDK1 can directly catalyze tubulin β phosphorylation, preventing its incorporation to microtubules. During apoptosis, increased CDK1 activity and other CDKs have been detected, suggesting that they could act as important regulators in the modifications of the microtubule cytoskeleton during the apoptotic process and the formation of AMN. CDK1 activity may also be responsible for actinomyosin ring contraction at the onset of apoptosis since previous studies have shown it can activate MLC kinase by phosphorylation. However, in PC12 cells, tubulin depolymerization at the onset of apoptosis has been associated

with tubulin deacetylation, activation of a PP2A-like phosphatase and dephosphorylation of Tau protein. However, both hypotheses are not mutually exclusive and that PP2A may regulate a cdc25 phosphatase which in turn dephosphorylates and activates CDKs.

## 4. Apoptotic microtubules during the execution phase of apoptosis

AMN formation in the execution phase of apoptosis has been reported in many cell lines (H460, A431, HeLa cells, primary human fibroblasts, and pig LLCPK-1$\alpha$ cells) and in response to a variety of apoptotic inducers such as camptothecin (CPT), staurosporine, anisomycin, UV irradiation, TRAIL (TNF-related apoptosis-inducing ligand), and serum withdrawal. In addition, AMN has also been observed in enucleated cells and apoptotic bodies. These findings suggest that AMN is a critical player in the genetically programmed process of apoptosis.

Topologically, AMN is organized in the proximity of the plasma membrane, forming a cortical structure that gives a typical "cocoon" like form, which delimits most of the intracellular contents including the fragmented nuclei and active caspases. Furthermore, apoptotic microtubules extend from the body of the cell as slender spikes, suggesting its structural role in maintaining the cell morphology during the execution phase of apoptosis. This spatial organization of the AMN might give some clues about its functional role during the execution phase of apoptosis. This arrangement also suggests a kind of cortical barrier or cocoon-like structure that may act to preserve plasma membrane integrity and/or as a structural barrier for the degradation reactions inside the cell. Other functions of apoptotic microtubules have been associated with the process of apoptotic body formation by helping to sustain the peripheral localization of chromatin within surface blebs and by facilitating cell fragmentation.

## 5. AMN visualization by live cell imaging

To exclude the possibility that the structures of the AMN could be due to artifacts during the fixation in the immunofluorescence microscopy protocol, AMN formation has been also studied by live cell imaging in pig epithelial cells (LLCPK-1$\alpha$) expressing GFP-tubulin and A431 cells expressing YFP-tubulin. In untreated interphase cells, microtubules are organized in long tubular polymers found throughout the cytoplasm growing from a central microtubule organizing center (MTOC) that corresponds to the localization of the centrosome. In cells undergoing apoptosis, this radial network organization is no longer preserved but is replaced by a cortical rearrangement of microtubules bundles enclosing the whole intracellular compartment corresponding to the AMN previously showed in fixed cells by immunofluorescence microscopy. Time-lapse imaging studies showed that the interphase microtubule network was depolymerized as the cells rounded-up in the early stages of the execution phase of apoptosis. However, microtubules were soon reorganized beneath plasma membrane as cells began retracting, forming the characteristic cortical cocoon-like structure of the AMN.

Under physiological conditions the cytoskeleton aids to maintain plasma membrane integrity. Changes to the cytoskeletal network could therefore alter membrane permeability. During the

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execution phase of apoptosis, the cortical actin network and intermediate filaments, which normally give support to plasma membrane, become depolymerized. In this situation, tubulin repolymerization that forms the AMN would be the only cytoskeletal component present in the apoptotic cell for preserving both plasma membrane integrity and cell shape. The organization of the AMN beneath plasma membrane surrounding the whole cellular volume suggests that tubulin reorganization in the execution phase of apoptosis might have a protective role helping to maintain plasma membrane integrity and thus delaying the transition to secondary necrosis. In fact, it has been reported that AMN was observed in all genuine apoptotic cells but was disrupted in cells in secondary necrosis. Furthermore, colchicine treatment, which disrupts apoptotic microtubules, increases cell permeability and the release of the intracellular marker lactate dehydrogenase (LDH). In addition to a purely structural role, AMN disorganization by colchicine treatment might facilitate the caspase cleavage of important proteins localized in the plasma membrane such as calcium channels and cellular cortex proteins such as fodrin (spectrin $\alpha$ II), which could in turn accelerate secondary necrosis by inducing ionic disturbances and cellular breakdown.

## 6. AMN formation

As mention above, formation of AMN is a biphasic process: first, during the early phase, interphase microtubules rapidly depolymerized but these were soon replaced by extensive bundles of closely packed, new tubulin polymers after actin and intermediate filaments are disassembled.

The microtubule depolymerization phase correlated with the loss of peripheral centrosomal $\gamma$-tubulin, suggesting that the two events may be linked. Notably, although the core centrioles remain essentially intact throughout apoptosis, they are unlikely to direct the formation of the novel apoptotic microtubule array because this is not assembled with radial pattern and instead appears randomly throughout the peripheral cytoplasm. The mechanisms responsible for centrosome disruption and indeed for initial microtubule disassembly remain undetermined. One possibility is that certain pericentriolar proteins are cleaved by caspases but to our knowledge none has been identified as a caspase target. Interestingly, it has been demonstrated that the minus-end-directed motor cytoplasmic dynein is essential for the centrosomal localization of pericentrin and $\gamma$-tubulin in healthy cells. Cytoplasmic dynein function is arrested during the execution phase by caspase cleavage of the intermediate chains. Therefore, one possible explanation is that this reduces the concentration of pericentrin and $\gamma$-tubulin at the centrosome, thereby abrogating its capacity to nucleate microtubules.

What stimulates the reassembly of microtubules in the execution phase remains uncertain. AMN assembly takes place in the absence of $\gamma$-tubulin discrete complex, suggesting that the formation of AMN is produced by other unknown mechanism.

Although they are tightly bundled, apoptotic microtubules remain dynamic – as judged by time-lapse imaging of the plus-end tracking protein EB1 – suggesting that their assembly is regulated. AMN reorganization of the execution phase of apoptosis occurs even in the presence

of caspase inhibitors. However, it has been postulated that active caspases may cleave the C-terminal regulatory regions of tubulins, which increases their ability to polymerize and thus facilitate the formation of apoptotic microtubules.

Jon Lane's group has reported that active, GTP-bound Ran is indeed required to support apoptotic microtubule assembly and that release of RanGTP into the apoptotic cytoplasm serves as a trigger for microtubule nucleation. They showed that the RanGTP-activated spindle-assembly factor, TPX2 (targeting protein for Xklp2), escapes from the nucleus during the execution phase and associates with apoptotic microtubule bundles. Consequently, silencing TPX2 expression by siRNA abrogates apoptotic microtubule assembly. They propose that formation of the apoptotic microtubule array shares several features in common with mitotic and meiotic spindle assembly with a particular dependence upon RanGTP and the microtubule-binding protein TPX2. Together, these observations suggest that, like mitotic and meiotic cells, apoptotic cells utilize the RanGTPase pathway to stimulate the coordinated assembly of a specialized microtubule network. Although AMN lacks the morphological and functional precision of the spindle apparatus, it nevertheless represents an important example of regulated, non-centrosomal microtubule assembly and organization and further highlights that the apoptotic execution phase should be recognized as a dynamic, tightly controlled process of cellular demise.

In another approach, the study of apoptotic microtubules components has revealed that in addition to the expected alpha and beta tubulin subunits, they intensively recruit other microtubule-associated proteins (MAP) such as MAP-4. These findings may be interesting for elucidating the role of MAPs in AMN nucleation. Given previous evidence linking MAP4 with microtubule nucleation, bundling, and stabilization, this protein could play an important role in AMN formation and maintenance.

## 7. Intracellular calcium chelation disrupts AMN and increases the permeability of plasma membrane

The elevation of intracellular calcium plays a pivotal role in the induction of the biochemical processes that characterize the execution phase of apoptosis. Among these changes, an early translocation of phosphatidylserine to the outer leaflet of the cellular membrane seems to be a key step in apoptosis, which has been shown to depend on caspase-3 activity and cytosolic $Ca^{2+}$ concentration. The resulting exposure of phosphatidylserine in the plasma membrane surface may serve as a "eat me" signal that triggers phagocytosis by macrophages.

Intracellular calcium levels are slightly elevated in genuine apoptotic cells with AMN. This slight elevation of intracellular calcium may play a role in the nucleation of the AMN because treatment with EGTA-AM that causes intracellular calcium chelation impairs AMN formation. These observations are consistent with a model whereby a slight increase in intracellular calcium, favored by the absence of actin and intermediate filament networks, triggers the reorganization of apoptotic microtubules in the execution phase of apoptosis.

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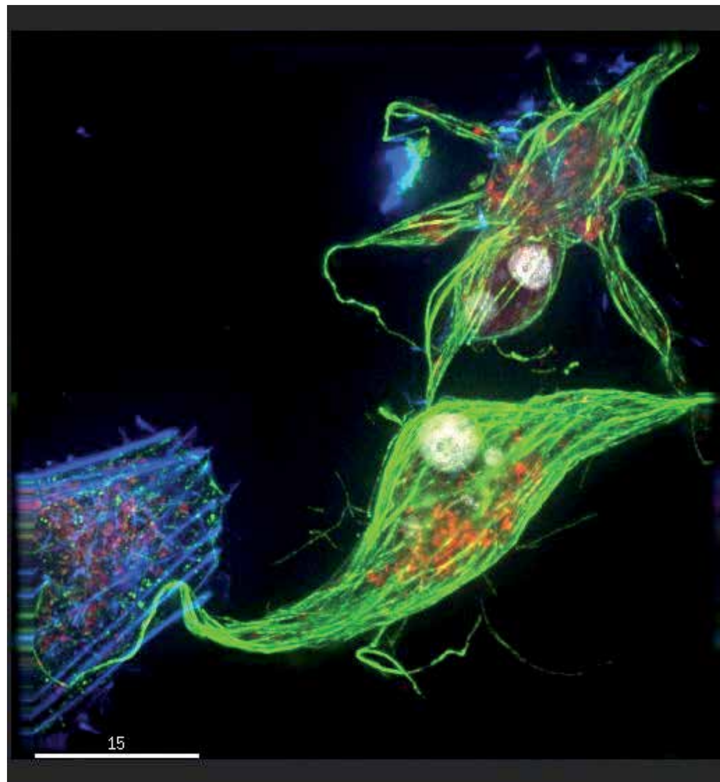


**Figure 2.** Human lung cancer apoptotic cells, H460: Green: anti-tubulin, AMN. Blue: actin. Red: Mitotracker, mitochondria; White: Hoechst, nuclei.

## 8. AMN disruption increases the permeability of plasma membrane

Given the critical role of apoptotic microtubules to maintain apoptotic cell integrity, any physical or chemical interference with AMN formation or stability might influence the process of genuine apoptosis and can induce a sort of derailed apoptosis with the release of toxic intracellular compounds that can have many pathological consequences in the context of multicellular organisms. Thus, as previously mentioned, the incubation of apoptotic cells with colchicine, an agent that depolymerizes microtubules, causes AMN depolymerization and increases plasma membrane permeability. In contrast, microtubules stabilization by taxol prevents both AMN disorganization and plasma membrane permeability.

Hypothermia or cold storage is widely used to protect cells and tissues against injurious processes. However, cold exposure can be deleterious for apoptotic cells given that microtubules which are essential to prevent plasma membrane permeability and secondary necrosis are cold labile. In contrast, AMN stabilization may preserve apoptotic cell integrity and prevents secondary necrosis.

Microtubules result from the polymerization of tubulin dimers in protofilaments that associate through lateral contacts. Microtubules are dynamic structures alternating growing and shrinking phases ended by catastrophes and rescues, respectively. *In vitro*, microtubule dynamics are under the control of the tubulin concentration and numerous other physico-chemical parameters. Among them, temperature plays a crucial role as microtubules depolymerize upon a temperature shift from 37°C to 4°C. This could be due to the modification of different dynamics parameters, especially the increase of catastrophe and the disappearance of rescue events at such temperatures. Because low temperatures depolymerize microtubules, cold exposition of apoptotic cells and re-warming to 37ºC leads to AMN disorganization and secondary necrosis. In contrast, apoptotic microtubules' stabilization by taxol prevents AMN disruption and secondary necrosis after cold/warming exposition.

Furthermore, apoptotic microtubules' depolymerization by cold/warming exposure was associated with less-efficient removal of dead cells and increased production of pro-inflammatory cytokines by macrophages. Taken together, these results indicate that temperature has an essential role for the correct execution of the apoptotic process. Furthermore, cold exposition of apoptotic cells impairs the proper phosphatidylserine externalization and interaction with macrophages, indicating that temperature is also critical for the efficient clearance of apoptotic cells. Consistent with a role of AMN for proper phosphatidylserine exposure, phagocytosis of apoptotic cells with stabilized microtubules by taxol coincided with high phosphatidylserine externalization while it was reduced when apoptotic microtubules were absent by cold/warming exposure and phosphatidylserine externalization was low, confirming previous results published by Moss *et al.* (2006). These data also suggest that the induction of secondary necrosis in apoptotic cells by cold/warming exposure is capable of inducing inflammation through the increased production of pro-inflammatory cytokines such as IL-1β and TNF-$\alpha$.

Therefore, cold or hypothermia although often used as a means of reducing cellular alterations or injury during periods of storage or transport of biological material, may induce secondary necrosis in cells already undergoing apoptosis.

These findings can be relevant in order to preserve apoptotic cells by cold storage and avoid the toxic and pro-inflammatory events induced by secondary necrosis.

## 9. Apoptotic microtubules delimit an active-caspase free area in the cellular cortex

AMN indeed may work as physical barrier impeding active caspases to access and cleave critical proteins in the cellular cortex and plasma membrane, which are essential for plasma membrane integrity (Figure 3). AMN disorganization in apoptotic cells by a short incubation with colchicine allowed caspase-mediated cleavage of cell cortex and plasma membrane proteins such as $\alpha$-spectrin, paxilin, Focal Adhesion Kinase (FAK), E-cadherin, plasma membrane $Ca^{2+}$ ATPase-4 (PMCA-4), $Na^{+}/Ca^{2+}$ exchanger (NCX), integrin β4, and $Na^{+}/K^{+}$ pump subunit β. These events were associated with increased cell permeability, calcium and sodium influx, and bioenergetics collapse, which in turn precipitate secondary necrosis. The essential

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role of caspase-mediated cleavage of cortical and plasma membrane proteins after AMN disassembly was confirmed because the addition of both colchicine and Z-VAD (a pan-caspase inhibitor) blocked protein cleavage and significantly prevented plasma membrane permeability, cell detachment, LDH release, calcium and sodium overload, and bioenergetics failure.

Under physiological conditions, plasma membrane cytoskeleton supports plasma membrane. The significance of this supporting cytoskeleton network has been mainly demonstrated in erythrocytes in which deficiencies or defects in the cytoskeletal proteins spectrin or spectrin-associated proteins were associated with increased fragility and lysis of plasma membrane. Changes in the cytoskeletal network beneath the plasma membrane (as $\alpha$-spectrin cleavage by caspases) may contribute to increase membrane permeability during the transition to secondary necrosis. Thus, spectrin-deficient spherocytes had decreased membrane mechanical stability, which probably contributes to cell lysis. It is thought that the spectrin skeleton acts universally to support the otherwise mechanically vulnerable cell surface bilayer. One way that membrane skeleton/bilayer interactions have been demonstrated is through the physiology of mechano-susceptible ion channels (channel whose gating is altered by abnormally high bilayer tension). These initially unresponsive channels become progressively more mechano-responsive under stretch and chemical reagents damage of the plasma membrane skeleton. The conclusion of these studies is that the intact membrane skeleton is mechano-protective and its perturbation may increase the ion permeability of the plasma membrane.

Likewise, focal adhesions are large, dynamic protein complexes through which the cytoskeleton of a cell interacts with the extracellular matrix. When cells adhere to the extracellular matrix, integrin receptors initiate signals to recruit more integrins and other cytoskeleton proteins (such as talin, tensin, vinculin, zyxin, and actinin), adapters (such as paxillin, Crk-associate substrate (p130CAS)1, and Crk), and kinases (such as FAK and Src) to their cytoplasmic tails, forming a “focal adhesion complex”. Focal adhesions provide not only mechanical support to cells through the connection with the actin cytoskeleton and mechanically couple the cell with the extracellular matrix but also signals necessary for anchorage-dependent cellular responses such as proliferation, migration, and inhibition of anoikis, a type of apoptosis induced by cell detachment. Integrins, heterodimers formed by one beta and one alpha subunits, interact with extracellular proteins via short amino acid sequences, such as the RGD (found in proteins such as fibronectin, laminin, or vitronectin) or the DGEA and GFOGER found in collagen.

Hydrolysis of “focal adhesion complex” proteins by caspases after AMN depolymerization could break the membrane-cytoskeleton linkage and decrease the physical support leading to cell detachment. It has been reported that FAK, integrin β4, and paxilin are cleaved in apoptotic cells when AMN was disorganized by colchicine treatment. Furthermore, this cleavage is a caspase-dependent process because it was blocked by z-VAD. These results suggest that disruption of FAK, integrin β4, and paxilin may contribute to cell detachment and the morphological changes observed in apoptotic cells undergoing secondary necrosis.

In agreement with these results, it has been demonstrated that E-cadherins are also cellular cortex proteins targeted by caspases when AMN is depolymerized. Cadherins are transmembrane glycoproteins involved in cell–cell adherence. Recent developments indicate that

classical cadherins may act as adherence-activated signaling receptors. Previously, it has been showed that cadherins are also targeted during apoptosis. Specific cell–cell and cell–matrix contacts regulate cell growth in epithelial cells and disruption of these contacts induces apoptotic cell death. According to these observations, caspase-mediated cleavage of E-cadherins and the loss of cell–cell contacts are likely to represent an important process during the extrusion of apoptotic cells undergoing secondary necrosis.

The $Na^+/K^+$-ATPase ($Na^+/K^+$-pump) acts as an electrogenic ion transporter in the plasma membrane whose primary role is to maintain high intracellular $K^+$ and low intracellular $Na^+$ concentrations. Each cycle of $Na^+/K^+$- ATPase enzyme pumps three Na+ ions out of the cell, moves two $K^+$ ions into the cell, and uses 1 ATP. Dysfunction of the $Na^+/K^+$-pump results in depletion of intracellular $K^+$, accumulation of intracellular $Na^+$ and, consequently, leads to membrane depolarization. Secondary, $Na^+/K^+$-pump failure increases intracellular free $Ca^{2+}$ ($[Ca^{2+}]i$) due to activation of voltage-gated $Ca^{2+}$ channels and reversed operation of the $Na^+$-$Ca^{2+}$ exchanger (NCX). Previous studies have shown that the intracellular $Na^+$ concentration increases prior to a loss of plasma membrane integrity and that cell shrinkage in apoptotic Jurkat cells is accompanied by a net efflux of ions due to an inactivation of $Na^+/K^+$-ATPase. The latter authors also observed that $Na^+/K^+$-ATPase subunits were degraded in populations with reduced volume during apoptosis. Because active cellular volume regulation requires $Na^+/K^+$-ATPase activity, both events may act synergistically in the induction of cell shrinkage. The $Na^+$-$K^+$-ATPase is composed of two subunits. The $\alpha$-subunit (~113 kD) is the catalytic subunit that binds ATP and both sodium and potassium ions and also contains the phosphorylation site. On the other hand, the smaller β-subunit (~35 kDa glycoprotein) is absolutely essential in facilitating the plasma membrane localization and $\alpha$-subunit activation. Studies on purified enzyme also suggested that both subunits were essential for activity because any efforts to separate $\alpha$ and β resulted in inactive enzyme. It has been shown that $Na^+/K^+$-ATPase β-subunits but not $\alpha$-subunits were cleaved by caspases after AMN depolymerization, which may contribute to ionic imbalance and increase plasma membrane permeability. Degradation of $Na^+/K^+$-ATPase β-subunit associated with mitochondria and plasma membrane depolarization during apoptosis has been previously reported. Consistent with this hypothesis, sodium levels were notably increased after AMN depolymerization by colchicine and partially restored when AMN was disorganized but caspases were blocked by z-VAD.

Changes in cytosolic calcium have an essential role during apoptosis by triggering the activation of $Ca^{2+}$-dependent processes thereby inducing global intracellular and morphological modifications including phosphatidylserine externalization. However, AMN disruption by colchicine allows the caspase-mediated cleavage of key proteins involved in calcium extrusion such as PMCA-4 and NCX and, consequently, provokes calcium overload. PMCAs are vital caspase substrates for the regulated subprogram leading to secondary necrosis. Cells expressing PMCA-4 mutants that lack the caspase cleavage site(s) prevent calcium influx during apoptosis and notably delay secondary necrosis. Furthermore, the $Na^+/Ca^{2+}$ transporter (NCX) also participates in calcium efflux in addition to PMCAs. NCX has a low calcium affinity but high calcium transporting activity, which is required to rapidly eject large amounts of calcium. While the NCX contribution in regulating resting cytosolic calcium may be less

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important than that of PMCAs, its function may avoid calcium overload in cells undergoing apoptosis. It has been reported that both PMC-4 and NCX are cleaved by caspases when AMN is depolymerized by colchicine treatment and suggest that inactivation of plasma membrane calcium transporters is a relevant process leading apoptotic cells to secondary necrosis.

Like many other insults, increased cytosolic calcium can trigger either apoptosis or necrosis. The final result of cell death is probably controlled by the concentration of cytoplasmic calcium. Whereas low to moderate calcium levels (200–400 nM) induces apoptosis, higher concentration of calcium (>1 μM) is associated with necrosis. This may help to understand why an initial slight calcium increase is pro-apoptotic and favors AMN formation whereas the late calcium influx through the plasma membrane that cannot be expelled out of the cell is associated with secondary necrosis. Calcium overload can induce: 1) activation of calpains leading to more extensive disruption of the protein components of cytoskeleton, 2) activation of calcium-dependent phospholipases causing liberation of arachidonic acid and formation of lysophosphatides that alter membrane structures and disruption of membrane permeability with ensuing secondary necrosis and/or 3) Mitochondrial permeabilization and bioenergetics collapse.

## 10. Apoptotic cells with AMN enhance phosphatidylserine exposure and interactions with macrophages

Phagocytic clearance of apoptotic cells or efferocytosis consists of four main distinct steps: accumulation of professional phagocytes at the site where apoptotic cells are located, recognition of apoptotic cells through a number of binding molecules and receptors, engulfment by a unique uptake process and digestion of engulfed cells within phagocytes. The efficient phagocytosis of apoptotic cells by macrophages reduces the potential for an inflammatory response by ensuring that the dying cells are eliminated before their intracellular contents are released to the extracellular medium. Early apoptotic cells are targeted for phagocytosis through the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. The externalization of phosphatidylserine is an early event of apoptosis occurring while the plasma membrane remains intact and cells exclude membrane-impermeant dyes. Phosphatidylserine exposure has been reported to be a process that needs energy and depends on caspase activation but its mechanism is still not clearly understood. A combined effect of down-regulation of a phospholipid translocase activity and activation of a lipid scramblase may participate in phosphatidylserine translocation.

Consistent with a role of AMN for proper phosphatidylserine exposure, it has been shown that the phagocytosis of apoptotic cells with AMN is associated with high appearance of phosphatidylserine on the cell surface while it was reduced when AMN was depolymerized by colchicine treatment. Phosphatidylserine externalization and phagocytosis of apoptotic cells were restored when AMN was depolymerized in the presence of Z-VAD, suggesting that caspase-dependent plasma membrane permeabilization impairs proper phosphatidylserine externalization.

These findings confirm previous observations that in the absence of AMN (nocodazole treatment), the proportion of macrophages interacting with and engulfing apoptotic targets was markedly reduced compared with apoptotic cells with AMN. The ability of apoptotic cells to trigger their own engulfment by phagocytic cells before cell lysis is crucial to prevent tissue damage and inflammation associated with necrosis.

## 11. Apoptotic microtubules' organization and maintenance depend on high cellular ATP levels and energized mitochondria

Microtubule polymerization *in vivo* is an energy-dependent process since the tubulin dimer contains bound GTP and GDP and the polymerization *in vitro* requires the presence of GTP and ATP. Therefore, a hypothesis has been proposed that AMN polymerization is dependent on the bioenergetic status of apoptotic cells.

In a normal genuine apoptotic process, ATP levels must be kept high to allow all the active processes occurring in apoptosis to proceed including AMN formation and maintenance. Live cell imaging and *in vitro* experiments have shown that AMN was organized predominantly in apoptotic cells with energized mitochondria and, on the contrary, was absent in apoptotic cells with depolarized mitochondria. These results suggest that AMN depends on polarized mitochondria and high ATP levels. Live cell imaging in pig LLCPK-1$\alpha$ cells expressing GFP-tubulin also showed that AMN was maintained during the execution phase of apoptosis until a large mitochondria depolarization marked the onset of secondary necrosis. Overall, these results suggest that AMN is a genuine marker of apoptotic cells in the execution phase of apoptosis. In addition, mitochondria depolarization by using a mitochondrial uncoupler (FCCP) or mitochondrial inhibitors (rotenone, antimycin, and oligomycin) induced AMN disassembly that was associated with LDH release and increased calcium influx, indicating increased plasma membrane permeability. On the contrary, 2-deoxyglucose, an inhibitor of glycolysis, had no effect either on mitochondrial polarization, AMN organization or calcium influx. Furthermore, apoptotic microtubules' stabilization by taxol prevented both the increased calcium influx and mitochondrial depolarization. AMN stabilization by taxol prevented calcium influx even in the presence of mitochondrial depolarization by rotenone or FCCP. All together, these results indicate that apoptotic microtubules' stabilization is sufficient to preserve plasma permeability and cell integrity during the execution phase of apoptosis.

Currently, there is no doubt that apoptosis requires energy because it is a highly controlled process involving a number of ATP-dependent processes such as caspase activation, enzymatic hydrolysis of macromolecules, nuclear condensation, blebbing, and apoptotic body formation. Depletion of cellular ATP was found to cause shifting of the type of cell death from apoptotic cell death to necrotic cell death. Thus, it is now well established that intracellular ATP levels determine whether the cell dies by apoptosis or necrosis. Increased ATP levels during the execution phase of apoptosis may also explain why mitochondria can be in a hyperpolarized state. In fact, it has been suggested that mitochondrial hyperpolarization is the consequence of high intracellular ATP levels. Thus, high ATP levels during apoptosis have been shown by

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other investigators. In one of the works, the authors made continuous measurements of cytosolic ATP levels throughout the apoptotic process. Their results showed that ATP levels within cells undergoing apoptosis were maintained higher than in control cells even as caspase activation and DNA fragmentation were occurring during the final stages of apoptosis. Also, they suggest that elevation of the cytosolic ATP level is essential for the apoptotic cell death process. These authors found that ATP levels were maintained at a higher level than the control value for over 70 min. A similar long-lasting ATP increase was observed also for cell death induced by TNF$\alpha$/CHX. In accordance with these results, it has been reported that AMN maintenance and mitochondrial hyperpolarization persisted in an interval of 50-158 min during the execution phase of apoptosis.

But, how are apoptotic cells able to keep up mitochondria with a high polarized state? The mitochondrial membrane potential reflects the energy stored in the electrochemical gradient across the inner mitochondrial membrane, which, in turn, is used by FoF1-ATPase to convert ADT to ATP during oxidative phosphorylation. Early mitochondrial hyperpolarization during apoptosis has been described previously. However, the precise mechanism(s) of mitochondrial hyperpolarization during apoptosis is not completely understood. Mitochondrial hyperpolarization has been reported to be mediated by the activity of oligomycin-sensitive mitochondrial FoF1-ATPase. The FoF1 complex can pump protons in reverse from the matrix, across the inner membrane into the intermembrane space under circumstances of high [ATP]/[ADP] + [Pi] ratios. Under these conditions, the FoF1 complex consumes ATP resulting in extrusion of protons from mitochondria. The activity of oligomycin-sensitive mitochondrial FoF1-ATPase operating in reverse mode during apoptosis and neurodegeneration has also been reported previously. It has been suggested that reverse functioning of the FoF1-ATPase participates in cytoplasm acidification and ROS (reactive oxygen species) production during apoptosis. However, there are many reports demonstrating that dissipation of the mitochondrial membrane potential is a general feature of apoptosis irrespective of cell type and of the apoptotic stimuli. While there is controversy regarding the significance of mitochondrial membrane potential loss during apoptosis (e.g., whether this is a cause or an effect of outer membrane permeabilization), the outcomes are predictable. The drop of mitochondrial membrane potential during apoptosis is expected to induce an arrest of the import of most proteins synthesized in the cytosol, release of $Ca^{2+}$ and glutathione from the mitochondrial matrix, entrance of extracellular $Ca^{2+}$ from the medium (calcium influx), uncoupling of oxidative phosphorylation with cessation of ATP synthesis, oxidation of NAD(P)$H_2$ and glutathione, and, finally, hyperproduction of ROS by the uncoupled respiratory chain.

These contradictory findings (mitochondrial hyperpolarization and depolarization during apoptosis) can be explained as follows: after a period of genuine apoptosis during the execution phase of apoptosis characterized by mitochondrial hyperpolarization, AMN polymerization, and low calcium levels, apoptotic cells *in vitro* undergo a process of secondary necrosis characterized by mitochondrial depolarization, AMN disassembly, and high intracellular calcium levels, although maintaining some of the typical characteristics of apoptotic cells such as fragmented nuclei and residual caspase activity. Therefore, apoptotic cells can show mitochondrial hyperpolarization or mitochondrial depolarization depending on the time of

analysis. Interpretation of these changes can also be more difficult if the study is not made in a homogeneous synchronized population because examination of a population of apoptotic cells can show a mixed population of cells with mitochondrial hyperpolarization or depolarization. Another interesting conclusion of these works is that AMN disorganization in apoptotic cells seems to be a consequence of mitochondria depolarization rather that increased calcium influx.

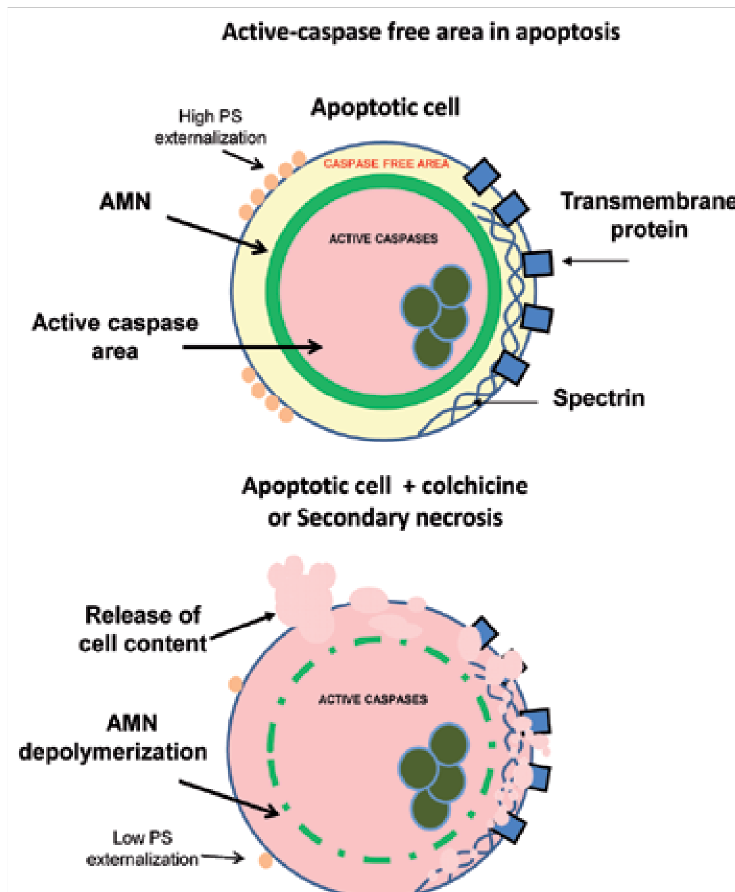


**Figure 3.** Scheme summarizing the main findings on AMN during the execution phase of apoptosis.

Furthermore, the observation of mitochondrial membrane potential fluctuations with cycles of hyperpolarization in the living cell imaging studies could reflect changes in the bioenergetic status of the apoptotic cell. When ATP levels are high, mitochondria are hyperpolarized by reverse flow of FoF1-ATPase that reduces ATP levels and permits the forward operation of FoF1-ATPase, which increases ATP levels again.

In summary, examining mitochondrial membrane potential during apoptosis, two features are striking: the cyclic changes of mitochondrial membrane potential associated with high ATP

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levels and the presence of AMN and the final mitochondrial depolarization coinciding with AMN disassembly and increased plasma permeability in the transition to secondary necrosis. These findings also support the hypothesis that AMN is essential for preserving plasma membrane permeability and, therefore, cell bioenergetics during the execution phase of apoptosis.

## 12. Apoptotic cells can be stabilized

Given that apoptotic cells maintain the integrity of the plasma membrane and cellular cortex, an innovative method aimed at the long-term stabilization and preservation of apoptotic cells has been developed. This method consists of the combined treatment of apoptotic cells with taxol, $Zn^{2+}$, and coenzyme $Q_{10}$ (CoQ). This experimental approach guarantees apoptotic cell integrity by preventing plasma membrane permeability and secondary necrosis for at least 96 hours in cell cultures.

The rationale for using this stabilizing combination is: a) the use of taxol, which as a microtubule stabilizing agent, prevents AMN depolymerization and subsequently the access of active caspases to the cellular cortex; b) the use of $Zn^{2+}$, which as a caspase inhibitor, avoids excessive degradation of cellular components and caspase-dependent cleavage of cellular cortex and plasma membrane proteins; and c) the use of CoQ, which as an antioxidant, protects against oxidative membrane damage which is increased in apoptotic cells.

Stabilized apoptotic cells can be seen as dying cells in which the cellular cortex and plasma membrane are maintained intact or alive. In a metaphorical sense, they can be considered as "living dead" or "zombie cells." Stabilized apoptotic cells have many of the typical hallmarks of genuine apoptosis such as plasma membrane impermeability, integrity of plasma membrane and cellular cortex proteins, low intracellular calcium levels, plasma membrane potential, phosphatidylserine exposure, and the ability of being engulfed by phagocytes.

Recently, interest in apoptosis research has increased considerably for a number of reasons including development of new treatments, cell culture technology, metabolic engineering of mammalian cells, and gene therapy. Furthermore, apoptotic cells play important roles in biomedical research because due to their characteristics, they are being widely used to evaluate the cytotoxic effects of various drugs by quantifying apoptotic cells by flow cytometry. However, this determination is often affected by the process of cell manipulation (cell harvesting, cell centrifugation, cell pipetting) required for flow cytometry assays, which may disrupt plasma membrane and lead apoptotic cells to secondary necrosis. Thus, accurate and reliable apoptosis quantifications are particularly difficult in adherent cell cultures. Stabilization of apoptotic cells before cell harvesting permits a more accurate and reliable quantification of the actual number of apoptotic cells or the correct measurement of biochemical parameters of genuine apoptotic cells such as mitochondrial membrane potential, intracellular calcium concentration, pH, caspase activity, and many others without the interference of plasma membrane disruption.

In addition, apoptotic cells are currently utilized in various forms of clinical treatments, primarily with the objective of inducing immunological tolerance in the recipient individual. The stabilization of apoptotic cells before administration may help to guarantee that the inoculated apoptotic cells retain their characteristic features until they are engulfed by phagocytes. The inoculation of stabilized apoptotic cells can be additionally utilized for delivering compounds of interest such as therapeutic proteins (for protein replacement therapy) or drugs to recipient macrophages.

There are processes of cell death which by their nature hinder the correct AMN formation (e.g., mitochondrial toxics and cold exposure) and subsequently apoptotic cells are not able to keep up plasma membrane integrity and cells undergo secondary necrosis that may cause serious adverse effects. Apoptotic cells' stabilization may permit the development of therapies for the correct formation and stabilization of AMN and thus promote a more physiological and controlled sort of cell death. Taking into account the above arguments, there is a need to find drugs and protocols for apoptotic cell stabilization.

## 13. Conclusion

In summary, AMN plays an essential role in the preservation of plasma membrane integrity during the execution phase of apoptosis. In the context of multicellular organisms, the most critical aspect of apoptosis is that cell death occurs without the release of potential pathogenic or harmful intracellular molecules, and without inflammation or injury to adjacent cells. In this manner, the primary role of AMN could be to guarantee that the dying cell is confined to prevent damage to the surrounding tissues and the potentially devastating consequences to processes such as tissue homeostasis and elimination of damaged cells. It is therefore essential to know the effects of different physical or chemical agents on the correct formation of this structure allowing us to better understand their effects on the immune system response and the potential adverse reactions.

Furthermore, apoptotic cells can be stabilized for accurate detection and quantification of apoptosis in cultured cells. Stabilization of apoptotic cells might likewise permit a more secure administration of apoptotic cells in clinical applications and open new alternatives for the functional reconstruction of apoptotic cells for longer preservation.

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Chapter 15

# Steroidal Saponins and Cell Death in Cancer

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Additional information is available at the end of the chapter

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**Abstract**

Steroidal saponins are natural glycosidic compounds of amphiphilic character. Their diverse biological activities are directly related to the variability of their structural constitutive frameworks, aglycones, and sugars. Several studies have demonstrated the therapeutic potential of steroidal saponins by their capacity to induce programmed cell death in different tumor cell lines. The process of cell death is required to maintain cellular and tissular homeostasis; it has been established that disturbances in the balance between cellular proliferation and cell death lead to several pathologies, including cancer. The antitumor activity of steroidal saponins has been intensely studied allowing elucidation of their different molecular mechanisms of action; this knowledge is crucial to the establishment of new therapeutic strategies against cancer.

**Keywords:** Steroidal saponins, cancer, cell death, apoptosis, cytotoxicity

## 1. Introduction

Saponins are a broad group of glycosides widely distributed in higher order terrestrial plants, and in lower marine organisms. They include a diverse group of compounds containing a steroidal or triterpenoid aglycone and one or more sugar chains [1]. Steroidal saponins are present almost exclusively in monocotyledonous angiosperms, not only in the families of *Dioscoreaceae, Asparagaceae, Liliaceae*, and *Amaryllidaceae* but also in the dicotyledonous *Solanaceae*. Triterpenoid saponins are more common in dicotyledonous angiosperms (e.g., the families *Caryophyllaceae, Quillajaceae, Sapindaceae*) [2]. Steroidal saponins are less common than triterpenoid saponins. Usually, glycosteroidal alkaloids are included in the very large alkaloid group.

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Mankind has used, for thousands of years, many saponin-containing plants as soaps. Saponins have an amphiphilic character and as soaps, they are surface-active compounds and produce micelles. They have a wide spectrum of uses; in ancient folk medicine, they have been used as venoms, hemolytes, antimicrobials, and anti-inflammatories. Saponins are responsible for numerous biological effects in traditional Chinese and Japanese medicines. New uses are in the cosmetic and pharmaceutical industries, as starting materials in the semisynthesis of many high-cost products. The latter are difficult to produce through total synthesis due to their great structural complexity and numerous chiral centers. The foaming property of saponins in water resulted in the coining of the word saponin (from Latin *sapo*, soap). Properties and pharmacological activities of saponins were described in great detail in 1927, before a single saponin had been fully characterized [1].

Steroidal saponins have a wide range of pharmacological applications, including use as expectorants and to inhibit platelet aggregation, and also have hemolytic, insecticidal, anti-inflammatory, antitumor, antidiabetic, antifungal/antiyeast, antibacterial, antiparasitic, antihyperlipidemic, and anti-oxidative properties, among others [3]. Taking into account the above applications, the physiological role of saponins in animals and plants has been related to their defense systems. One of the first uses in the health field was made in the immune system, since they activate the immune response to antigens, functioning as adjuvants that improve the effectiveness of orally administered vaccines by facilitating the absorption of large molecules [4]. Later studies have allowed identifying saponins as inductors of cell death by means of several molecular mechanisms.

## 2. Chemical characteristics of saponins

Structurally, saponins are composed of a lipid-soluble aglycone that consists of a steroidal or triterpenoid skeleton and a water-soluble moiety, composed of sugar residues. The latter can differ in the type and amount of cyclic carbohydrates. The natural properties of saponins allow them to be dissolved in water where they form colloidal solutions that foam upon shaking [5]. The structure of saponins derived from plant sources are different from those found in animals. The same structural difference is observed in steroidal or triterpenoid saponins. In general, their water solubility depends on their sugar moiety number [6].

The triterpenoid aglycone consists of a skeleton of 30 carbon atoms, showing in general a pentacyclic structure. In triterpene saponins, ten main classes are found: dammaranes, tirucallanes, curcubitanes, lanostanes (all with a four six-membered ring skeleton), cycloartanes (possessing a cyclopropane attached to a four six-membered ring skeleton), lupanes and hopanes (in which a cyclopentane ring is attached to a four six-membered ring skeleton), oleananes, taraxasteranes, and ursanes (composed by a five six-membered ring skeleton) [7]. In all cases, several skeletons have been found to undergo ring cleavage (seco-skeletons), homologation (homo-skeletons), degradation (nor-skeletons), or rearrangements (abeo-skeletons).

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All steroidal saponins contain a 27 carbon atom aglycone skeleton and are classified in three main subclasses: spirostan, furostan, and cholestane saponins [8]. Spirostan saponins contain an aglycone that is composed of four six-membered and two five-membered rings (named as A, B, C, D, E, and F-rings, Figure 1); aglycones of furostan saponins possess only A, B, C, D, and E rings (three six-membered and two five-membered rings), while aglycones of cholestane saponins have only the tetracyclic A, B, C, and D system (three six-membered and one five-membered rings). Biosynthetically, spirostans and furostans derive from a cholestane skeleton through selective oxidation pathways.

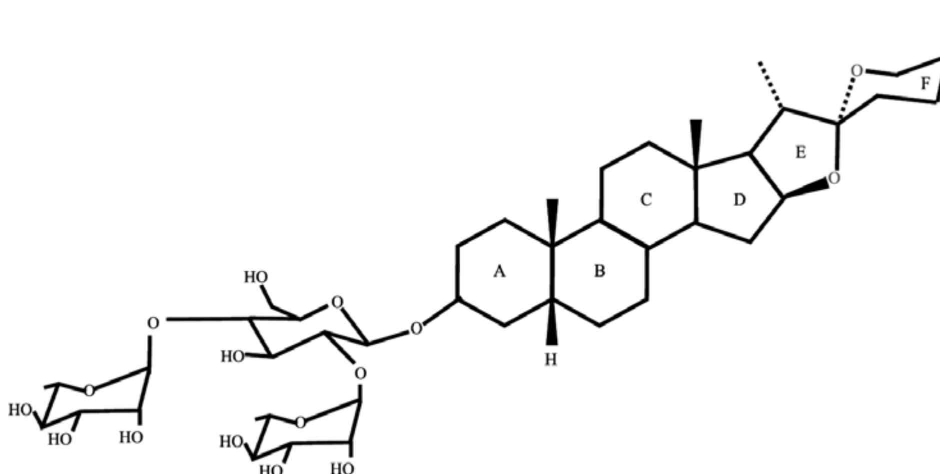


**Figure 1.** Structure of a spirostan saponin. Typical hexacyclic ABCDEF-ring system.

The steroidal saponin dioscin (Figure 7) had a huge importance as the favorite starting material in the steroid industry. A first transformation, an enzymatic or acidic hydrolysis, produced its aglycone diosgenin, and then modification of the diosgenin homoallylic enol and the spiroketal moieties gave progestagens, androstagens, corticosteroids, and some other important biological compounds. It is also possible to obtain a partial hydrolysis working under smooth-controlled conditions. Dioscin, and its chemically related saponins polyphyllin D and balanitins have a remarkable anticancer activity. These monodesmosidic saponins present oligosaccharide chains in which the first sugar, β-D-glucopyranose, is attached to the diosgenin C-3 position, and this in turn is substituted via its 2-OH and 4-OH positions. Commonly, $\alpha$-L-rhamnopyranose, $\alpha$-L-arabinofuranose, and other sugars constitute their oligosugar chains [9].

## 3. Diverse biological activities of saponins

As previously mentioned, steroidal saponins have an extensive variety of biological activities (Figure 2), including the absorption of cholesterol from the small intestine [10]. Mice treated with saponins from the plant *Tribulus terrestris* L. showed total cholesterol reduction in the

liver and total serum [11], and hyperlipidemia was prevented. This control of cholesterol occurs through interaction with saponins, producing insoluble complexes that are excreted in bile, thus inhibiting entero-hepatic cholesterol recycling and reducing blood cholesterol levels (reviewed in [12]).

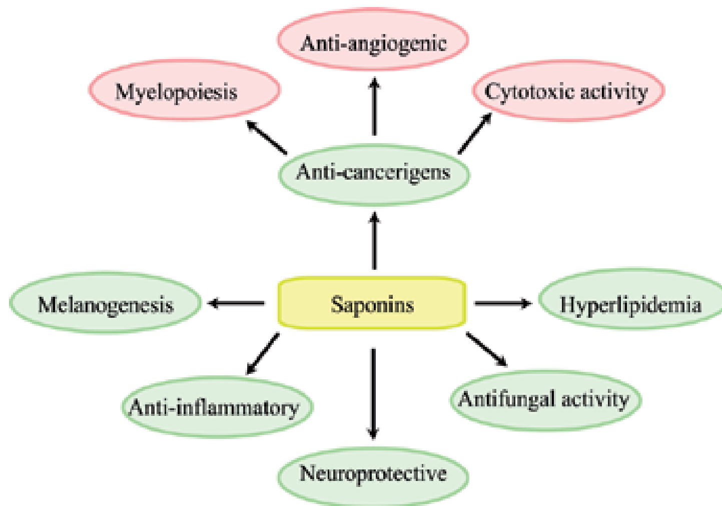


**Figure 2.** Biological activities of saponins.

### 3.1. Steroidal saponins from ginseng

Steroidal saponins are present in different types of plants. Ginseng (the root of *Panax ginseng*, C.A. Mey.) contains a series of ginsenosides that belong to the family of steroidal saponins, and these exhibit several biological properties (Figure 3). Ginsenosides are chemically structured by a skeleton consisting of four trans/anti-fusioned rings with modifications related to the type and number of sugar moieties and the attachment sites of the hydroxyl groups [13]. The two major components of the ginsenoside family are protopanaxadiol and protopanaxatriol [14]. The sugar moieties in the protopanaxadiol and protopanaxatriol are attached to the 3-position and 6-position of a dammarane-type triterpene, respectively (Figure 4). The protopanaxadiols include ginsenoside Rb1, Rb2, Rc, and Rd, while protopanaxatriols include ginsenoside Re, Rf, and Rg1.

Several reports indicate that each ginsenoside has distinct biological effects; it has been shown that purified ginsenoside protopanaxadiol Rb1 has a neuroprotective effect on PC12 (rat adrenal pheochromocytoma cell line) cells inhibiting the cell death by decreasing both the amount of active caspase-3 as well as DNA fragmentation, and increasing the amount of the anti-apoptotic Bcl-xL protein [15]. Besides acting as a neuroprotective, Rb1 has anti-angiogenic function inhibiting the process of new blood vessel formation [16], as well as an anti-inflammatory function [17].

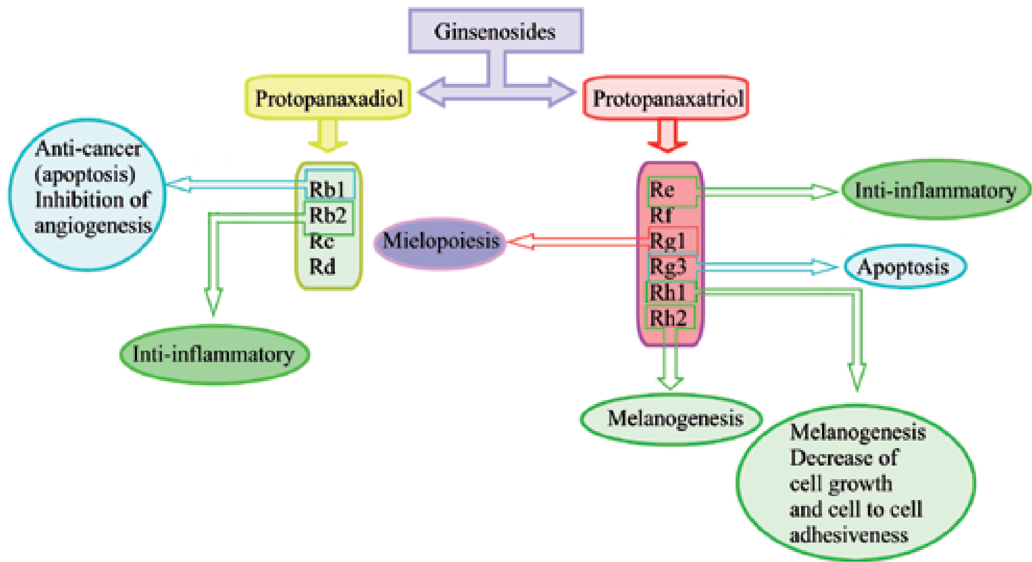


**Figure 3.** The two major groups of ginsenosides and their specific biological functions.

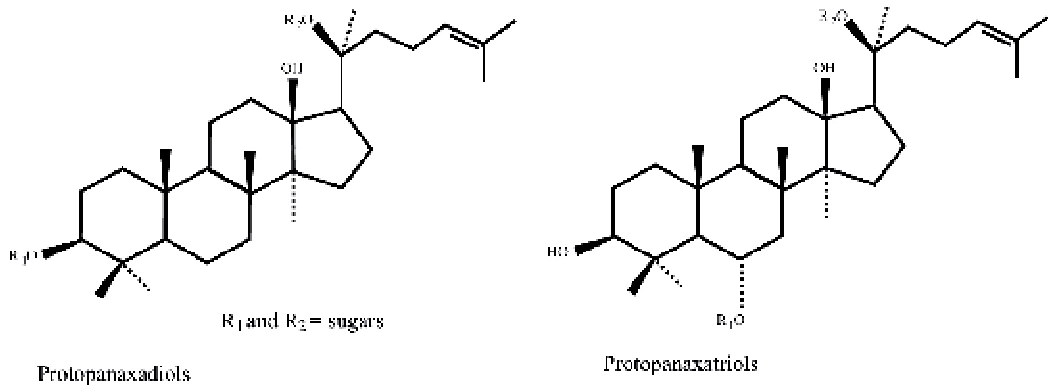


**Figure 4.** Structure of ginsenosides protopanaxadiols and protopanaxatriols.

The ginsenosides of the protopanaxatriol group have been shown to have several functions, some of which are similar to those exhibited by the protopanaxadiols. An anti-inflammatory effect has also has been shown by using the Re member [18]. With respect to the promotion of cell death by this group, it has been observed that Rg3 induces cell death in hepatocellular carcinoma cells in a selective form, since it does not affect normal cells [19]. The treatment of several types of tumors implies the use of chemotherapeutic agents that possess secondary reactions such as myelosupression. It has been shown that the protopanaxadiol Rg1 enhances myelopoiesis in vitro and reconstitutes bone marrow after myelosuppression treatment in mice [20].

The structural composition of saponins is important for their biological activity. Ginsenosides Rh1 and Rh2 (members of the panaxytriol group) are extracted from the root of *Panax ginseng*, but their specific activity changes according to the sugar moiety binding site. Clear examples of this are Rh1 and Rh2, which both stimulate melanogenesis, but only Rh1 decreases cell growth and cell-to-cell adhesiveness [6].

The use of botanical products containing steroidal saponins showed that the saponins present in *Panax ginseng* and *Panax quinquefolius* L. affect the central nervous system by acting on the hypothalamic–pituitary–adrenal and hypothalamic–pituitary–gonadal axes (reviewed in [21]). Observations have also revealed that ginseng extract inhibits the growth of several types of tumors, such as B16 melanoma cells [22] and hepatoma cells [23].

### 3.2. Steroidal saponins derive from diverse plant sources

With respect to other saponins not derived from ginsenosides, several studies have demonstrated the therapeutic potential of steroidal saponins with antifungal activity. This effect has been attributed to both their individual aglycone moieties and the number and structure of their monosaccharide units. Certain pathologies associated with immunocompromised diseases as opportunistic fungal infections have been treated with the steroidal saponins C-27, which are composed of a C-27 aglycone moiety and a sugar chain with one or more monosaccharides [8]. This response could be the reason that saponins are capable of inducing a nonspecific immune response having an immunomodulatory activity by stimulating both cell-mediated and humoral immune responses [24]. Steroidal saponins denominated SC-1-SC-6 are present in *Solanum chrysotrichum* Schltdl. The SC-2 saponin is a potent antimycotic [25].

An important biological function of saponins is their capacity to induce cell death by means of programmed or nonprogrammed routes. The effect that these compounds exert inside the tumor cell has been widely evaluated, providing evidence that they could be used as agents to control cell proliferation. Steroidal saponins derived from *Withania somnifera* (L.) Dunal (Ashwagandha) have shown antiproliferative activity in rat C6 glioma cell lines [26]. Saponins can act at different cellular levels and are capable of forming pores in lipid bilayers increasing the cellular permeability, thus enabling the uptake of molecules that would otherwise be excluded and produce toxic stimuli [27]. The toxic activity shown by saponins has allowed them to be considered as possible therapeutic agents, but it is important to contemplate the possible side effects caused by their toxicity, since drugs have a dual effect; i.e., while they control certain pathologies and contribute to curing particular diseases, they can have harmful side effects that cause adverse consequences and symptoms in the body. Recent reports suggest that some saponins exhibit antiproliferative and apoptotic activity and act selectively without presenting cytotoxicity [28–32]. These findings are generating great interest in these compounds as therapeutic agents for treating cancer.

## 4. Antitumor activity of saponins

Cancer includes a group of complex genetic diseases that affect aged cells. Carcinogenesis is a multi-step molecular process induced by genetic and epigenetic changes that disrupt the

balance between cell proliferation, apoptosis, differentiation, senescence, and the pathways that control these cellular processes (see review in [33]).

Different types of programmed cell death are known, including apoptosis and autophagy (Figure 5). Both processes are complex and are regulated by different enzymatic activities. Apoptosis is morphologically characterized by cellular shrinkage, DNA fragmentation, and the formation of cellular fragments surrounded by a cytoplasmic membrane termed apoptotic bodies [34]. The enzymatic activity in apoptosis is developed by the caspases that are the proteases responsible for the morphological changes [35]. Autophagic cell death is characterized by an exacerbated formation of autophagic vesicles and an increased lysosomal activity [36, 37]. The hallmark of the programmed cell death processes is the absence of an immunologic response that takes place in the accidental cell death known as necrosis.

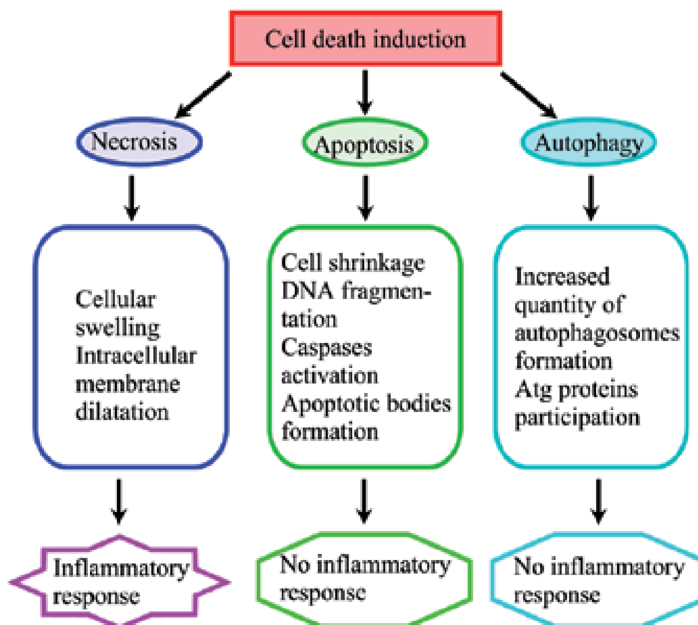


**Figure 5.** Characteristics of the necrosis, apoptosis, and autophagic cell death. Each cell death has its own morphological and biochemical properties.

Numerous studies using in vitro and in vivo models have been conducted to evaluate the antitumor activity of various saponins (Figure 6), including triterpene and steroidal saponins and diosgenin. Different cancer types have been treated with plant extracts that have a high quantity of steroidal saponins, as well as with isolated or synthetic steroidal saponins.

Several such assays have used plant extracts in different tumor cells. A recurrent model in the evaluation of steroidal saponins is the hepatocellular carcinoma cells HepG2. It has been shown that in the extract obtained from *Asparagus officinalis* L. whose components include steroidal saponins [38, 39], polysaccharides, and flavonoids, the steroidal saponins are the major

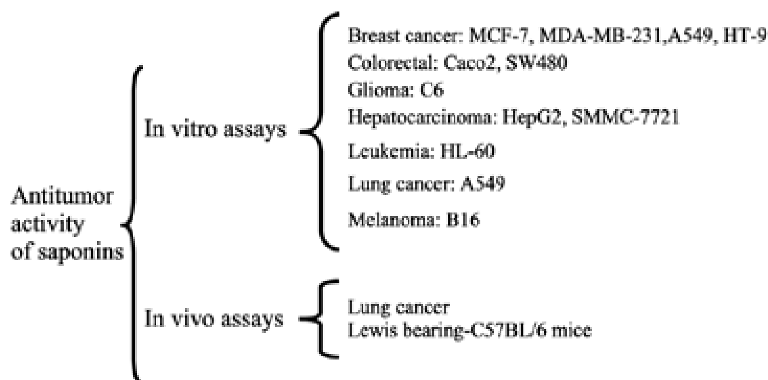


**Figure 6.** In vitro and in situ assays to evaluate the antitumor activity of saponins inside different types of cancer.

components responsible for antitumor activity, since in HepG2 cells they induce apoptosis [40]. Steroidal saponins isolated from *Paris polyphylla* Sm. – specifically three pennogenin steroidal saponins (monodesmosidic glucosides) – exhibit significant antiproliferation activities against HepG2 cells, inducing apoptosis [7].

Several strategies have been used to control cancer cell proliferation, including inducing cell death or cytotoxic effect on cancer cells; however, new strategies could be planned to allow more efficient chemotherapy treatments. Steroidal saponins from *Trillium tschonoskii* Maxim. reverse multidrug resistance in HepG2 and R-HepG2 cells, and significantly enhance chemo-sensitization to doxorubicin, thus reducing tumor formation in vivo [41].

In colon cancer cells, the anticancer effect of several plants containing saponins has been observed inside human colon cancer cell lines. *Allium flavum* L. is a plant traditionally used as a spice, but steroidal saponins from the whole plant have presented cytotoxic effects in the human colon cancer, SW480 cells, and their derived metastatic SW620 cells (a derivate of mesenteric lymph node metastasis) [42]. *Allium macrostemon* Bunge has an active steroidal saponin called macrostemonoside A (MSS.A), which suppresses cell growth in Caco2 and SW480 human colorectal cancer cell lines in two ways: by arresting the cell cycle and by inducing apoptosis [43].

Breast cancer is one of the most common malignancies in women and the second leading cause of cancer deaths [44]. It has been reported that the extract of *Dillenia suffruticosa* (Griff) Martelli shows high antioxidant and cytotoxic activities in several cancer cell breast lines, such as MCF-7, MDA-MB-231, A549, and HT-9 [45]. It has been proposed that this cytotoxic property may be due to the presence of saponins, triterpenes, tannins, and polyphenolic compounds in the extract [54]. Tor et al. (2014) [66] showed that the ethyl acetate extract exerts a series of effects in the cancer cell breast line MCF-7, including non-phase specific cell cycle arrest and apoptosis by increasing ROS (reactive oxygen species). The biological activity of *Fagonia indica* Burn. f. var. schweinfurthii *Hadidi* (family *Zygophyllaceae*) is attributed to saponin glycosides that exert a necrotic effect on the breast cancer cell lines MCF-7 and MDA-MB-468 cancer cells [46].

The antitumor effect of saponins has also been demonstrated in ovarian cancer cell lines. The principal constituents of *Rhizoma Paridis* (a stem of *Paris polyphylla* Sm. var. *chinensis* (Franch.) H. Hara) are steroidal saponins such as formosanin C (PSII). PSII has shown an antitumor effect in ovarian cancer cells, and studies have demonstrated that it achieved its effect by activating several mechanisms, including cell-cycle arrest and apoptosis [47].

The antitumor effect of saponins in lung cancer has been reported in both in vivo and in vitro models. The immunomodulatory role of the steroidal saponins obtained from *P. polyphylla* var. *chinensis* has been demonstrated in lung cancer using Lewis bearing-C57BL/6 mice [48]. These steroidal saponins induce apoptosis cell death in A549 lung cancer cells.

Intense research into the anticancer effects of steroidal saponins has led to the discovery of new compounds whose properties could be improved. Researchers have isolated compounds that have been used individually or in combination to induce cell death in different cancer type cells. The steroidal saponins (25 R)-5$\alpha$-spirostan-3β,6β-diol 3-O-β-D-glucopyranosyl-(1-4)-[β-L-arabinopyranosyl-(1-6)]-β-D-glucopyranoside was cytotoxic for A549, HeLa, and LAC human cancer cell lines [49]. The steroidal saponin tupichinin A, together with seven known compounds isolated from rhizomes of *Tupistra chinensis* Baker, showed potent cytotoxicity against the cancer cell lines HL-60, SMMC-7721, A-549, MCF-7, and SW480 [50], while the mixed saponins balanitin-6 and -7 showed anti-tumor activity in both in vivo and in vitro systems [51]. This is evidence of the broad range and the various mechanisms of action of saponins.

Some steroidal saponins have been extensively evaluated such that each compound has an antitumor effect on different cancer types, and examples of this are dioscin and diosgenine (Figure 7). Dioscin provokes G2/M phase arrest and apoptosis in human gastric cancer SGC-7901 cells [52].

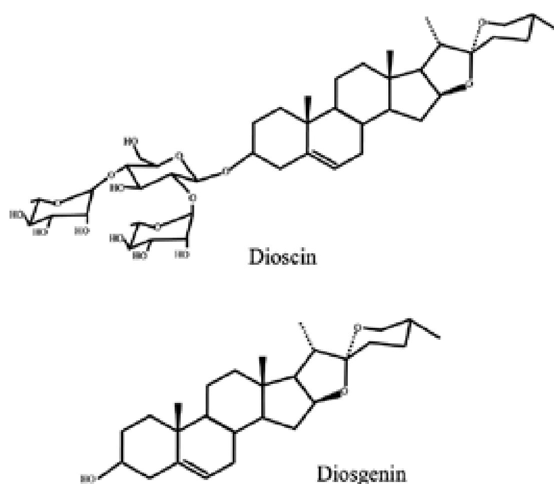


**Figure 7.** Structures of dioscin and diosgenin.

Diosgenin is an aglycone of steroidal saponins that exhibits antiproliferative and pro-apoptotic activities on cancer cells in vitro. Cancer metastasis involves the migration of cancer cells from the primary tumor. In this process, the matrix metalloproteinases are the main proteases that participate in tumor cell migration, spreading, tissue invasion, and metastasis [53]. Multiple studies have demonstrated the role of diosgenin as an anticarcinogenic factor, and have shown that diosgenin was able to inhibit metastasis in vitro in human prostate cancer PC-3 cells [54]. In B16 mouse melanoma cells, treatment with diosgenin inhibits melanogenesis by a mechanism of action that involves the PI3K signaling pathway [55].

Steroidal saponins have been widely used to control tumor expansion; however, it is important to take into account the characteristics of this kind of cell control, since some steroidal saponins isolated from diverse plants have not only antiproliferative characteristics but also undesirable effects. Da Silva et al. (2002) [56], for example, evaluated the anti-inflammatory activity of a steroidal saponin isolated from the leaves of *Agave attenuata* Salm-Dyck (*Asparagaceae*), but found that while it showed anti-inflammatory activity, it also has a hemolytic effect.

Steroidal saponins have been incorporated into in vivo models to control the cancer process. A recent study using nude mice bearing human hepatocellular carcinoma showed the effect of the pennogenyl saponins (PS1 and PS2) isolated from *Rhizoma Paridis*. Results from Chen et al. (2014) [57] suggest that these saponins inhibit the progression of hepatocellular carcinoma by inducing apoptosis, activating both routes of caspase activation – extrinsic and intrinsic – and inhibiting cell proliferation.

In vitro assays have provided important information on the anticancer properties of diverse steroidal saponins. However, it is important to note the need to increase the number of such experiments in order to corroborate the effects identified, while remembering that in vivo systems involve several parameters that influence the effect of the different plant extracts and the compounds isolated or synthesized from them. These include gastrointestinal absorption, kinetics, bio-availability, tissue distribution, the systemic circulation pathway, catabolism, and excretion [46].

## 5. Molecular cell death mechanisms of saponins in cancer cells

A desired feature in a compound used in cancer treatment is that it has the ability to remove cells in a regulated manner, with the least possible side effects. This requirement means activating the programmed cell death routes.

Previously, we mentioned that a hallmark of programed cell death is the absence of an anti-inflammatory response, which is avoided by the conservation of the cellular membrane until the late phase of the process. Apoptosis can be activated by means of two routes: the extrinsic path that involves the participation of a cytoplasmic membrane receptor, and the intrinsic route that implies the delivery of pro-apoptotic proteins by the mitochondria (Figure 8). The molecular mechanism of the apoptosis is characterized by the participation of the proteases named caspases, which can be activated by the mentioned routes. Caspases are responsible

for the morphological changes during the apoptotic process since they are able to depolymerize the cytoskeleton components.

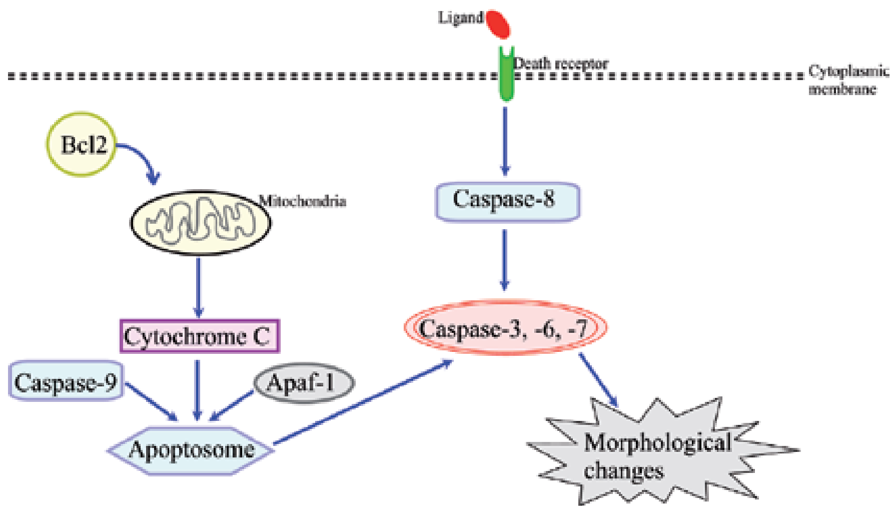


**Figure 8.** Intrinsic and extrinsic routes of activation of caspases. Mitochondrial activation involves the cytochrome-C delivering from the mitochondria to form a complex composed by the caspase-9 and the Apaf-1, which in turn will activate the executer caspases -3, -6, or -7. Extrinsic route implies the activation of a death receptor in the cytoplasmic membrane by means of a ligand; this process will activate the initiator caspase-8, which in turn will activate the executer caspase-3, -6, or -7. The activation of the executer caspases provokes the morphological changes related to the apoptotic process.

The molecular activity of different saponins is attributed to their structural composition. It has been demonstrated that the heterosugar moiety causes heteropolarity of steroidal saponins, leading to different membrane permeability and selectivity in the bioactivity of the compounds [7]. Saponins act at different molecular levels inside cells, and this can lead to several modifications in cellular organization.

Saponins can induce the extrinsic route of apoptosis by activating the cell death receptors present in the cell cytoplasmic membrane. As previously mentioned, the ginsenosids are used as treatment against cancer events, and several reports have identified the molecular mechanism by which they exert their apoptotic function. The 20(s)-ginsenoside Rg3 renders HCC cells are more susceptible to TRAIL (i.e., TNF-related apoptosis-inducing ligand-induced apoptosis) by upregulating DR5 (death receptor 5). An important characteristic of this system to induce cell death is that this regulation does not affect normal cells [19].

Steroidal saponins are able to promote cell death acting inside different cellular organelles, which in turn promote the release of some molecules which promote apoptosis. Some of the targets of saponins are the mitochondria and the endoplasmic reticulum; the collapse of mitochondrial potential induces a release of cytochrome-C, activating the intrinsic apoptotic pathway [58]. The endoplasmic reticulum stress triggers the release of calcium; this delivery

induces the mitochondrial apoptotic pathway [59]. It has been shown that saponins obtained from *Asparagus officinalis* induce mitochondrial and caspase-dependent apoptosis, increasing intracellular levels of reactive-oxygen-species (ROS) and calcium [40]. In the same way, the saponin dioscin induces apoptosis by the intrinsic route increasing intracellular calcium and as a consequence the mitochondria deliver pro-apoptotic proteins to active the caspases [52]. The molecule OSW-1 (3β,16β,17α-trihydroxycholest-5-en-22-one 16-*O*-{*O*-[2-*O*-(4-methoxy-benzoyl)-β-D-xylopyranosyl]-(1 → 3)-2-*O*-acetyl-α-L-arabinopyranoside}, isolated from bulbs of *Ornithogalum saundersiae* Baker [60], triggers an elevation in cytosolic and mitochondrial calcium concentrations. This increased calcium level then activates apoptotic factors via the interaction of OSW-1 with the endoplasmic reticulum ATPase and its endoplasmic reticulum chaperone GRP78, which is involved in endoplasmic reticulum stress responses [61]. The timosaponins are steroidal saponins of the coprostane type that have pro-apoptotic and protective autophagy functions in HeLa cells [62]. Endoplasmic reticulum stress is induced by several factors, including the accumulation of misfolded proteins. Elimination of the sections of the endoplasmic reticulum that accumulate defective proteins is carried out by the autophagy process. Autophagy is regulated by mTOR (Ser/Thr kinase target of rapamycin) (reviewed in [63]), such that when this kinase is inhibited autophagy is activated. Timosaponin TAIII induces cell death in tumor cells but not normal ones, by inducing apoptosis via endoplasmic reticulum stress, and can inhibit mTORC1 [64] while exerting its effect selectively. In fact, in vitro treatments with several saponins have shown effects on the endoplasmic reticulum.

The antitumoral effects of the saponin dioscin have been studied widely, leading to the suggestion that the results of dioscin-induced molecular expression may have a cell-type-specific correlation. It has been reported that dioscin has the ability to induce apoptosis by activating the intrinsic or extrinsic route of apoptosis execution. In HeLa cells, a cell line derived from a human cervical carcinoma, the dioscin activates the intrinsic route since this inhibits the anti-apoptotic protein Bcl-2, and activates the pro-apoptotic proteins caspase-9 and caspase-3 in HeLa cells [65]. The activation of caspase-8 is not present in HeLa cells treated with ioscin, indicating that the extrinsic routes of caspase activation do not participate in HeLa cells treated with ioscin. On the contrary, the same ioscin provokes the extrinsic apoptosis activation in human myeloma leukemia HL-60 cells, inducing FasL and FADD expression, caspase-8 activation, and Bid truncation [66], demonstrating the activation of apoptosis by cell death receptor. The vast majority of the saponins that perform this pro-apoptotic role exert their function by activating the intrinsic apoptosis pathway.

Apoptosis is a complex mechanism leading to cellular elimination, in which several factors are involved, including those that regulate the transcription process. NF-kappaB is a transcriptional factor that normally remains in an inactive form in the cytoplasm. But once activated, it is released from its inhibitor and translocated from the cytoplasm to the nucleus. Inside the nucleus, it binds in the promoter region of several target genes related to cell proliferation, angiogenesis, and metastasis [40]. Diosgenin [(25*R*)-5-spirosten-3β-ol] is a steroidal sapogenin that inhibits the invasion of tumor cells when induced by TNF (tumor necrosis factor). The diosgenin inhibits the osteoclastogenesis induced by RANKL (receptor activator of nuclear factor kappa-B ligand) by inhibiting NF-kappaB and NF-kappaB-regulated gene products [67].

This activity suggests that saponins act at the molecular level by inhibiting NF-kappaB, blocking the expression of proliferation genes, and inducing apoptotic death through the intrinsic pathway and the participation of pro-apoptotic genes.

Apoptotic cell death can be triggered by the activation of different routes of signaling besides the caspases cascade. One of the responsive routes of signaling involves the mitogen-activated protein kinase (MAPK) family members. MAPKs are serine/threonine kinases that under certain stimuli phosphorylate specific substrates, regulating diverse cellular responses including apoptosis. The saponins present in plant extracts can induce several biochemical effects that impact critical enzymes involved in signal transduction pathways such as ERK 1/2 (extracellular signal-regulated kinase 1/2). One MAPK family member, PSII – specifically – modifies ERK activities and increases the level of active caspases [47]. It has been shown that plant extracts containing saponins exerted a cytotoxic effect by increasing oxidative stress that, in turn, activated Akt (protein kinase B, a serine/threonine kinase) [68]. Akt is one enzyme involved in cell proliferation, apoptosis, and angiogenesis. The high oxidative stress induced by saponin extracts also exerts its effect inside the p53 protein (tumor suppressor protein) and the p38 MAPK signaling pathway [68]. These effects lead to cell elimination and provide saponins with antiproliferative properties.

A morphological change characteristic of apoptosis is cellular shrinkage, which is a consequence of the cytoskeleton depolymerization caused by the action of active executor caspases such as caspase-3. The effect of saponins inside elements of the cytoskeleton, whose major structural components are the microtubule and actin filaments, has also been demonstrated. The mixed saponins, balanitin-6 and balanitin-7, affected the stability of the actin cytoskeleton by depleting ATP, thus exercising antitumor activity [51]. Cellular ATP depletion in diverse cell types provokes the change of the polymerized form of F-actin into a monomeric G-actin [69]. Actin polymerization of the F-actin form allows the cell to perform diverse functions, such as mitosis, movement, signaling transduction, and substance transportation. This means that it enables correct cellular functioning.

The population of cancer cells can be regulated either by inducing cell death or by inhibiting their proliferation. Several steroidal saponins obtained from diverse plants have demonstrated their effect by inhibiting the cellular cycle progression. The saponin ioscin causes cell cycle arrest by inhibiting cyclin B1 and CDK1 [52]; the same effect has been observed in the steroidal saponin PSII (formosanin C), which also caused cell-cycle arrest [47]. Cyclooxygenases (COXs) are enzymes active in the conversion of arachidonic acid into prostanoids, which are involved in apoptosis, inflammation, mitogenesis, and immunomodulation [70]. Of their two isoforms, COX-1 is present in a constitutive form, while COX-2 is an inducible form [43]. It has been shown that diosgenin eliminates COX-2 by promoting cell cycle arrest in the G1 phase and inducing apoptotic cell death [70].

The impact of saponins at the molecular level involves altering the levels of energy required for adequate cell physiology, disrupting transduction pathway signaling, and triggering the cell death process (Figure 9).

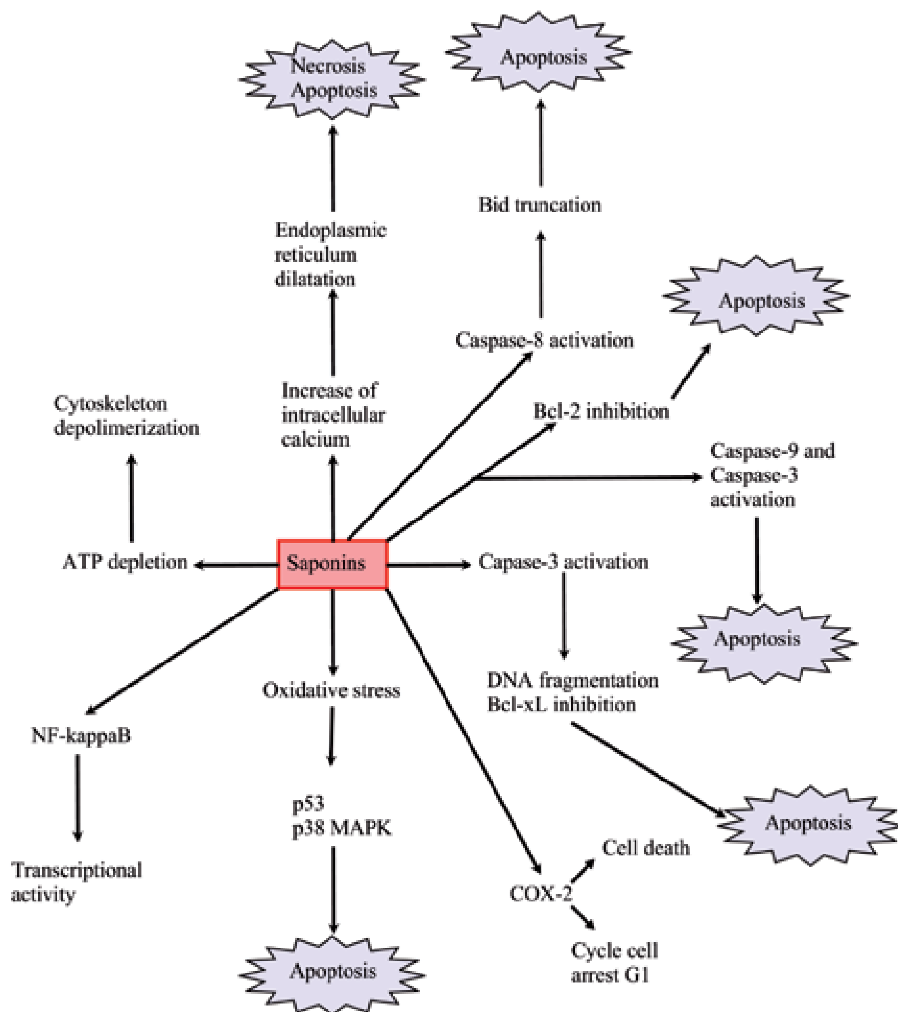


**Figure 9.** Different molecular cell death pathways activated by saponins. Saponins are able to induce the intrinsic and the extrinsic pathway of activation of the apoptosis cell death. In the same form, it influences inside different molecular levels including inside transcription factors as NF-kappaB as well as in the route of signaling of the MAPK. The correct polymerization of the cytoskeleton components is affected by the saponins, since this provokes a depletion of ATP inhibiting the correct polymerization of actin. In several occasions, the steroidal saponins not only induce the cell death process but also can inhibit the cell cycle progression.

## 6. Conclusions

Steroidal saponins are compounds that manifest antiproliferative activity and necrotic induction, and promote apoptotic or autophagic cell death in tumor cells. The important biological property of these compounds is their capacity to induce programmed cell death

http://dx.doi.org/10.5772/61438

(apoptosis) in different tumor cell lines. In view of the fact that the compounds used in anticancer treatments are unspecific and inefficient in terminal patients and may have side effects stemming from their cytotoxic activity, research groups are looking for new compounds with antiproliferative activity that are noncytotoxic and have selective action. This aspect is relevant because it implies that the side effects related to cytotoxic activity could be reduced quite significantly. The knowledge of different molecular mechanisms of cell death triggered by saponins is of great importance because these compounds have been shown to have significant potential as antitumor agents, and may be apt for use in treating cancers, with important cost-benefit advantages and reduced side effects.

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# Cell Death Induction by Targeting Tumor Metabolism

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**Abstract**

Over the last century, a broader interest in the topic of tumor metabolism has emerged. From the 1920s onward, when Otto Warburg proposed increased aerobic glycolysis of tumor cells, a deeper understanding has established that tumor cells have an altered metabolism which is directly linked to cancer progression. It was soon discovered that not only do environmental changes lead to alterations in metabolism but that oncogenes have a profound influence in these alterations. They not only induce nutrient uptake and synthesis of proteins and DNA but can lead to a switch toward glycolysis, which identifies them as a major player in tumor metabolism. These observations have raised the interest to target metabolic pathways for cancer therapy and, interestingly, some of the first discovered chemotherapeutics target metabolic pathways and are still in clinic. Concerns that these targets will also affect normal cells has intensified research to understand how changes in tumor metabolism promote tumor growth and which enzymes and signaling pathways are involved. These observations led to the discovery of new targets and drugs that specifically affect tumor metabolism and can exploit the dependence of tumor cells on the metabolic changes.

**Keywords:** Tumor metabolism, glycolysis, AMPK, lipid metabolism, p53

## 1. Introduction

Already in the 1920s, Otto Warburg described that tumor cells typically use aerobic glycolysis for energy production rather than oxidative phosphorylation (OXPHOS) despite sufficient oxygen and the lesser yield of ATP. By now, altered tumor metabolism is recognized as a hallmark of cancer cells, which allows them to escape from the typical regulatory constraints that prevent normal cells from uncontrolled growth and proliferation. A number of theories have been proposed to explain this phenomenon, amongst them independence of oxygen especially in hypoxic areas, which often occur in tumors. Furthermore, tumor cells often have mutations in mitochondria, which could explain a shift toward glycolysis. Glucose and

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glutamine uptake also have the advantage that they can not only be used as ATP source but also as building blocks for essential metabolites required for uncontrolled growth (e.g., amino acids, nucleotide triphosphates, NADPH) [1, 2]. Glucose is used for the formation of nucleic acids via the pentose phosphate pathway and glycolytic intermediates are used for fatty acid biosynthesis. Therefore, highly proliferating tumor cells need to change different aspects of their metabolism to meet the high demand of energy in the form of ATP and secure the supply of the major classes of macromolecules: carbohydrates, proteins, lipids, and nucleic acids (Figure 1).

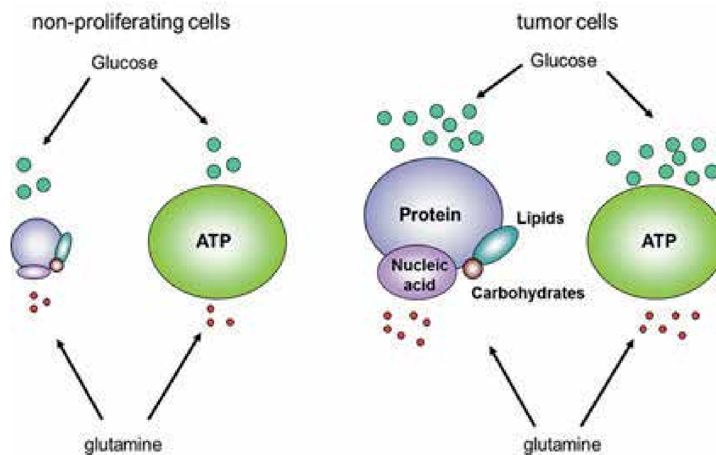


**Figure 1.** Tumor cells use glucose and glutamine not only for ATP production but also as source of essential metabolites. To ensure uncontrolled growth and proliferation, they have a much higher demand of nutrients, which is provided by aerobic glycolysis.

In recent years, it has also been revealed that the metabolic reprogramming is not necessarily induced by different metabolic requirements but also regulated by oncogenes and tumor suppressors. The PI3K/Akt pathway is often deregulated in tumor cells. Akt is one of the most important proteins in cells which have elevated glycolysis as it promotes the increased expression and membrane localization of the glucose receptor-1 (GLUT-1) and stimulates phosphofructokinase activity. It thus promotes augmented glucose uptake and increased glycolytic activity. The tumor suppressor p53, on the other hand, can shift tumor cells from glycolysis to oxidative phosphorylation [3, 4]. The major inducer and regulator of glycolysis is the hypoxia-inducible factor-1$\alpha$ (HIF1$\alpha$), a transcription factor that is often found in highly metastatic and neoplastic tumor cells and strongly promotes glycolysis.

Alterations in tumor metabolism are quite heterogeneous, which impedes the finding of a generalized target. The changes depend on the availability of nutrients, oxygen, and the pH, and in turn depend on the different tumor vasculature. Proliferation requires nutrient uptake, metabolite and DNA synthesis, and energy production. Thus, genetic alterations in signaling pathways that drive the cells to proliferate are often involved in changes of the tumor metabolism. But drugs that target, e.g., DNA synthesis to inhibit proliferation are also directed

against normal proliferating cells and therefore lack selectivity. Therefore, the systematic characterization of the metabolic pathways that differ in cancer cells is an ongoing challenge which must lead to the discovery of drugs that specifically target proteins or enzymes altered in tumors. Targeting tumor metabolism has become a promising field in cancer therapy but requires an in-depth understanding of the metabolic regulation and signaling pathways involved, which will be reviewed here.

## 2. Targeting tumor metabolism

Understanding the metabolic pathways altered in tumor cells targeting the resources or specific pathways that fuel deregulated tumor metabolism has shown to be an attractive strategy for cancer therapeutics. Hexokinase inhibitors like 2-deoxy-D-glucose have already shown promising results in preclinical studies but also dichloracetate, which targets pyruvate dehydrogenase kinase (PDK1); gemcitabine, which inhibits nucleic acid synthesis; or metformin, which induces the AMP-activated protein kinase (AMPK) are interesting compounds for targeting tumor metabolism. Interestingly, tumors with high glucose uptake detected by the 2-[18F]fluoro-2-deoxy-D-glucose–positron emission tomography (FDG–PET) scan show a worsened outcome [5], confirming the importance for drugs targeting tumor metabolism.

The complexity and tight regulation of the tumor metabolism raises the possibility to target multiple pathways, enzymes, and proteins, some of the most important ones of which will be addressed here.

### 2.1. Glycolysis

#### *2.1.1. Hexokinase*

Hexokinase catalyzes the first and rate-limiting step in glycolysis (see Figure 2) by the ATP-dependent phosphorylation of glucose to yield glucose-6-phosphate (G6P). There are four different hexokinase isoforms (HK1-4), which show different tissue distribution and enzyme activity. The high affinity kinases HK1 and HK2 are inhibited by excess G6P, are associated with the mitochondria, and implicated in cell survival [6]. Hexokinase-2 was shown to be highly expressed in tumor cells but only in a limited number of normal tissues and is partly responsible for the increased glycolytic activity of tumor cells. Hexokinase level could also be correlated with tumor stage and patient survival [7].

Due to the importance of hexokinase for the glycolytic flux, there exist a variety of inhibitors such as 2-deoxy-D-glucose (2DG), 3-bromopyruvate, and lonidamine, which showed promising effects in preclinical studies [8].

2DG is a glucose analogue that is phosphorylated by hexokinase to form 2DG phosphate, which cannot be further metabolized. Treatment of tumor cells with 2DG inhibits glycolysis, leading to ATP depletion, cell growth inhibition, and apoptosis [9]. 2DG has extensive metabolic effects and not only affects glycolysis but also OXPHOS. In normoxia, it can interfere with N-linked glycosylation and induce the unfolded protein response [10].

Despite promising activities *in vitro*, the effects of 2DG as a single agent *in vivo* and in clinical trials were a disappointment. This might be due to off-target effects like the activation of the PI3K pathway or the induction of pro-survival autophagy [11]. Furthermore, the dose needed for complete inhibition of glycolysis in patients showed severe side effects. In combination therapy, however, lower doses of 2DG, which are better tolerated, could improve the efficiency [12].

#### *2.1.2. Hypoxia-inducible factor-1α*

Tumor growth is associated with intratumoral hypoxia due to the lack of sufficient vascularization. The physiological consequence for the tumor to survive in this hostile environment is to increase angiogenesis to achieve adequate oxygen delivery, to adapt the metabolism by increasing glucose uptake, and switch to glycolysis for energy supply. All these actions are initiated by the transcription factor hypoxia-inducible factor-1$\alpha$ (HIF1$\alpha$), which is the major regulator of glycolysis and induces more than a hundred genes involved in metabolism [13]. Increased activity of HIF1$\alpha$ is known to correlate with poor patient outcome and, interestingly, some highly neoplastic tumors like renal cell carcinoma frequently carry a mutation that leads to a constitutive active HIF1$\alpha$. The best known action of HIF1$\alpha$ is the induction of VEGF (vasculature endothelial growth factor), which is required for angiogenesis [14, 15]. It also induces the expression of the glucose transporters GLUT-1 and -3, and activates a number of glycolytic enzymes like aldolase, phosphofructokinase, enolase, and lactate dehydrogenase. It furthermore activates pyruvate dehydrogenase-1 (PDK1) and, thereby, reduces the flow of pyruvate used by the TCA cycle, decreasing OXPHOS and further increasing glycolysis, as shown in Figure 2.

HIF1 is a heterodimer composed of the constitutive expressed HIF1β and the oxygen-sensitive HIF1$\alpha$. In the presence of oxygen, HIF1$\alpha$ is hydroxylated by prolyl hydroxylases on prolyl-residues, which are required for the binding of the ubiquitin E3 ligase Von-Hippel-Lindau (VHL). Binding of VHL results in the degradation of HIF1$\alpha$ by the proteasome. Under some circumstances like overexpression of the PI3K/Akt pathway or the induction of reactive oxygen and nitrogen species, HIF can be stabilized even under normoxia [16].

Due to the importance of HIF1$\alpha$ in tumor growth and survival and the poor outcome of patients with high levels of HIF1$\alpha$, targeting this protein seemed to be a promising option in tumor therapy. Interestingly, until now no specific inhibitors of HIF1$\alpha$ exist in the clinic, although there have been various efforts, and some experimental drugs exist, which inhibit transcription, translation, or DNA binding of HIF1$\alpha$ [17]. The most promising drugs inhibit HIF1$\alpha$ as a side effect; like rapamycin, which inhibits mTOR activity. The PI3K/Akt/mTOR pathway plays an important role for HIF1$\alpha$ translation and rapamycin as well as the PI3K inhibitor LY294002 have shown to be able to reduce HIF protein expression as well as expression of its target genes [18, 19].

The proteasome inhibitor bortezomib is another drug that was reported to inhibit adaption to hypoxia of tumors and to functionally inhibit HIF1$\alpha$. Bortezomib is approved for the treatment of multiple myeloma and, therefore, an interesting candidate for analyzing effects on HIF. It

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inhibits the expression of the HIF target genes VEGF and erythropoietin and the recruitment of p300 coactivator [20].

### 2.2. Signaling proteins and growth control elements

#### *2.2.1. PI3K/Akt*

The PI3K pathway is one of the most common altered signaling pathways in cancer. The main actor is the serine/threonine kinase Akt, which is able to activate many downstream targets involved in cell growth, survival, and cell cycle progression. A constitutive activation of Akt not only leads to strong pro-survival signals but also has a strong impact on tumor metabolism. Increased Akt signaling has been shown to directly correlate with increased glucose metabolism in a variety of tumor cells [21]. It supports aerobic glycolysis even in untransformed cells when overexpressed. Akt increases glucose uptake through promoting the translocation of the glucose receptor GLUT1 to the plasma membrane and also induces several glycolytic enzymes like hexokinase and phosphofructokinase. Furthermore, it mediates the increase of a variety of fatty acid- and cholesterol-synthesis enzymes. It promotes binding of the hexokinase to the mitochondrial membrane and thereby, like Bcl-2 or Bcl-X, increases mitochondrial membrane integrity and inhibits apoptosis [22]. Finally, Akt strongly induces signaling via mTOR by phosphorylating and inhibiting its negative regulator tuberous sclerosis complex 2 (TSC2). mTOR is a key metabolic checkpoint which leads to induction of protein and lipid biosynthesis when activated and, therefore, promotes cell growth. The importance of Akt in glucose metabolism is also supported by the fact that targeting the PI3K pathway in animal models and the use of kinase inhibitors in patients lead to a decrease in glucose uptake as measured by FDG-PET uptake [23, 24].

These observations lead to the assumption that inhibition of Akt would lead to inhibition of glycolysis and especially kill tumor cells that are dependent on a permanent supply of glucose. Using Akt inhibitors on tumor cells with aberrant PI3K signaling would, on the one hand, sensitize them to glucose-starvation-induced apoptosis as shown by Elstrom *et al*. (2004) [21] and, on the other hand, would open a new therapeutic window for PI3K inhibitors in cells dependent on aerobic glycolysis.

#### *2.2.2. AMPK*

The AMP-activated protein kinase (AMPK) belongs to a family of serine/threonine kinases and is highly conserved from yeast to mammals. It consists of a catalytic $\alpha$ subunit and regulatory $\beta$ and $\gamma$ subunits. It is the most important energy-sensing protein in the cell and activated by a number of metabolic or oncogenic stresses like glucose and nutrient deprivation or hypoxia. AMPK senses the levels of AMP and ATP and is activated by an increased AMP/ATP ratio. Upon activation, it increases catabolic processes that generate ATP-like fatty acid oxidation and glycolysis and inhibits anabolic processes that consume ATP-like protein and lipid synthesis [25] (shown in Figure 3). The dominant upstream kinase that regulates AMPK activity is the liver kinase B1 (LKB1), which is a known tumor suppressor. Loss-of-function mutations of this kinase were first discovered in Peutz-Jeghers syndrome, an autosomal

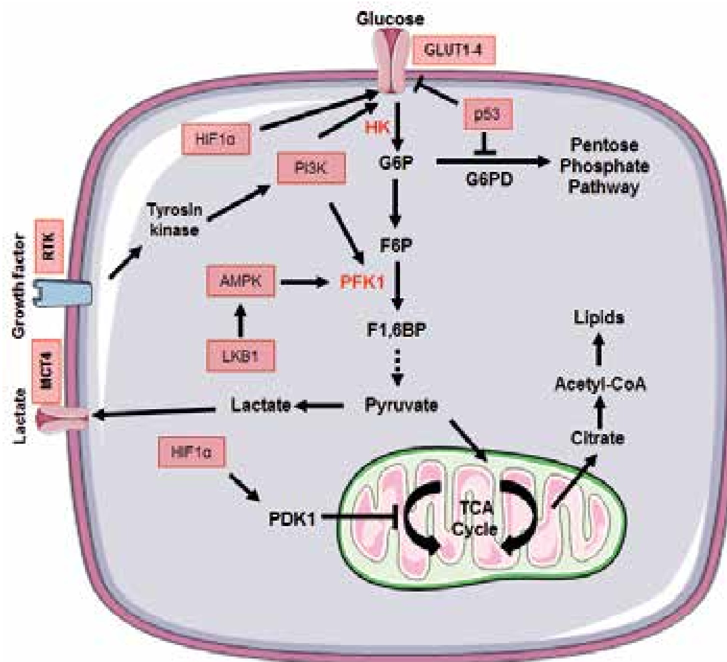


**Figure 2.** Simplified schema of tumor metabolism showing glycolysis, TCA cycle, lipid metabolism, and pentose phosphate pathway. Shown are proteins that are overexpressed or mutated to guarantee an increased glycolysis and decreased oxidative phosphorylation (HIF1$\alpha$, PI3K/Akt, p53). HK (hexokinase), PFK1 (phosphofructokinase-1), GLUT (glucose receptor), G6P (glucose-6-phosphate), PDK1 (pyruvate dehydrogenase kinase-1), TCA cycle (tricarboxylic acid cycle), RTK (receptor tyrosine kinase), MCT4 (monocarboxylate transporter-4).

dominant genetic disorder characterized amongst others by an increased risk of gastrointestinal adenocarcinoma [26]. Mutations of LKB1 were also found in several tumors like sporadic lung adenocarcinoma or cervical carcinoma. One of the major downstream targets of AMPK is mTOR, which induces protein synthesis. During energy stress, AMPK leads to an inhibition of mTOR on the level of TSC2 and consequently to an inhibition of protein synthesis. Interestingly, ablation of LKB1 abolishes this inhibitory effect [27].

Although decreased activity of AMPK was shown to promote tumor growth, in recent years, it has become evident that AMPK activation can also prevent tumor formation, especially under conditions of hypoxia and glucose deprivation. Deletion of AMPK$\alpha$1 synergizes with myc to promote lymphangiogenesis of B cell lymphoma, which supports the role as a tumor suppressor. In breast carcinoma, reduced AMPK phosphorylation inversely correlated with histological grade and axillary node metastasis [28]. Furthermore, AMPK was shown to inhibit cell proliferation by stabilization of p53 or regulation of cyclin-dependent kinase (CDK) inhibitors p21 and p27. But although activated AMPK can inhibit tumor cell proliferation, loss of AMPK is not sufficient to allow proliferation in the absence of nutrients. There are even reports that tumor cells lacking AMPK undergo apoptosis during metabolic stress and are

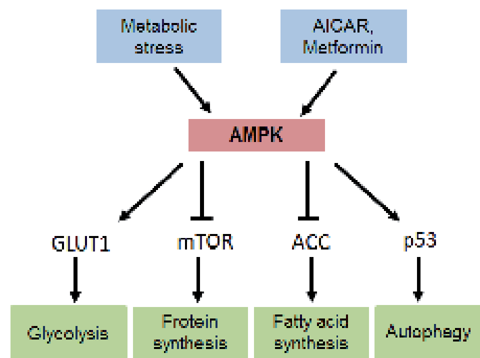


**Figure 3.** Major functions of AMPK activation. AMPK activation leads to an induction of glycolysis, fatty acid oxidation, and autophagy, and to an inhibition of protein synthesis.

resistant to oncogenic transformation [29]. On the other hand, pharmacological activators of AMPK like metformin, AICAR, or A769662 inhibited or delayed tumor formation in animals [30]. Faubert *et al*. (2013) could show that silencing AMPK promotes a metabolic shift to the Warburg effect with increased glucose uptake, glycolytic flux, and flow of carbon to the tricarboxylic cycle to fuel pathways of ATP production and biosynthesis [31]. The pro-apoptotic mechanisms of AMPK might be mainly associated with the inhibition mTOR. In line with this assumption, it was shown that AMPK activation by metformin or the AMP analogue AICAR correlated with mTOR inhibition in renal cell carcinoma [32].

Pro-survival mechanisms of AMPK can be partly explained by autophagy induction, which is mediated by p53, and maintenance of proliferative quiescence [33]. Therefore, the role of AMPK as an oncogene or tumor suppressor seems to be dependent on the degree of AMPK activation and duration of nutrient deprivation. Despite the promotion of AMPK agonists like metformin as anticancer treatment, there needs to be a deeper understanding of AMPK regulation in tumorigenesis to implement AMPK-targeting drugs into clinic.

### *2.2.3. p53*

The tumor suppressor protein p53 is the most frequent mutated gene in cancer. It is well known for its role in DNA damage repair and apoptosis induction after cellular stress. Depending on the stress signals, it either leads to growth arrest by inducing p21, Gadd45 or p48 or in response to severe stresses it induces genes involved in apoptosis (Puma, Bax, Fas) or senescence (p21). But it is emerging that it also plays a pivotal role in tumor metabolism by inhibiting glycolysis and switching tumor metabolism to OXPHOS and thus inducing apoptosis.

p53 has multiple functions in tumor metabolism and considering its role as tumor suppressor, it is not surprising that p53 counteracts metabolic changes associated with cancer growth: it transcriptionally represses the expression of the glucose transporters GLUT1 and GLUT4 and indirectly represses expression of GLUT3 via NFκB inhibition and, therefore, inhibits the first rate-limiting step in glycolysis [34]. At the third step of glycolysis, phosphofructokinase-1

(PFK1) is inhibited by various metabolites that indicate sufficient supply of energy like ATP, citrate, and lactate. AMP and fructose-2,6-bisphosphate (F26B), which indicate low energy, activate PFK1 and, thus, increase glycolytic flux. TP53-inducible glycolysis and apoptosis regulator (TIGAR) is an important protein activated by p53. It acts as a phosphatase and degrades F26B decreasing the activity of PFK1 and consequently lowers the glycolytic rate [4, 35]. There are also some other glycolytic players that are inhibited by p53-like expression of pyruvate dehydrogenase kinase 2 (PDK2), which leads to a decreased conversion from pyruvate to lactate. Inhibition of the glycolytic pathway on multiple levels would assume that glucose would be shuttled into the pentose phosphate pathway (PPP) but it was recently shown that p53 also inhibits PPP by binding and inhibiting glucose-6-phosphate dehydrogenase (G6PD), the enzyme that catalyzes the first step of the PPP [36]. Therefore, p53 can reduce the production of NADPH and ribose-5-phosphate, which are important for reactive oxygen defense and DNA synthesis.

p53 not only inhibits glycolysis but also enhances OXPHOS: it transcriptionally activates cytochrome c oxidase 2 (SCO2), which is required for the assembly of the cytochrome c oxidase complex (complex IV in the mitochondrial electron transport chain) [37] and induces the expression of AIF (apoptosis-inducing factor), which is important for OXPHOS, most likely by ensuring the proper assembly of mitochondrial respiratory complex I. Furthermore, p53 regulates mitochondrial DNA copy number and mitochondrial quality control by removing damaged mitochondria [38]. By these actions, p53 directly counteracts the Warburg effect and therefore opposes metabolic changes that are essential for malignant transformation.

The metabolic functions of p53 are emerging as critical for tumor suppression and apoptosis induction via targeting tumor metabolism. Nevertheless, the many different stress signals that activate different p53 responses are still not fully understood. Therefore, future studies to understand the molecular mechanisms that activate p53 and mediate the responses to metabolic stress need to be performed to finally exploit and manipulate them for tumor therapy.

### 2.3. Pathways

#### *2.3.1. Pentose phosphate pathway*

Glucose is the main energy source not only for tumor cells but for all organisms. It enters the cell via glucose transporters and is then phosphorylated by hexokinase to form glucose-6-phosphate (G6P). From this point, G6P can either enter glycolysis to produce energy in form of ATP or it is shuttled to the pentose phosphate pathway (PPP). There, it is either hydrogenated by G6PD (glucose-6-phosphate dehydrogenase) and further converted to yield ribulose 5-phosphate in the oxidative branch. Or it enters the nonoxidative branch, which is catalyzed by transketolase, and generates ribose-5-phosphate, which is a precursor of biomolecules like nucleotides and, therefore, important for DNA synthesis (see Figure 4). During the oxidative phase, $NADP^+$ acts as electron acceptor during the oxidative reactions and 2 molecules of NADPH are yielded. NADPH plays an important role in the protection of the cell from oxidative stress. Therefore, the PPP plays a pivotal role in reductive biosynthesis like lipid and

nucleotide synthesis and is essential for antioxidant defense. It is strongly connected with glycolysis and glucose is shuttled to the pathway where it is needed most. Historically, most attention was paid to glycolysis as it provides the energy for biosynthesis, but highly proliferating cells also need large amounts of lipids as energy storage and building blocks for membranes and nucleotides for DNA replication [39]. These needs are fulfilled by the PPP. In recent years, more attention was paid to changes in the pathway and effort is taken to find ways to target the PPP for tumor therapy.

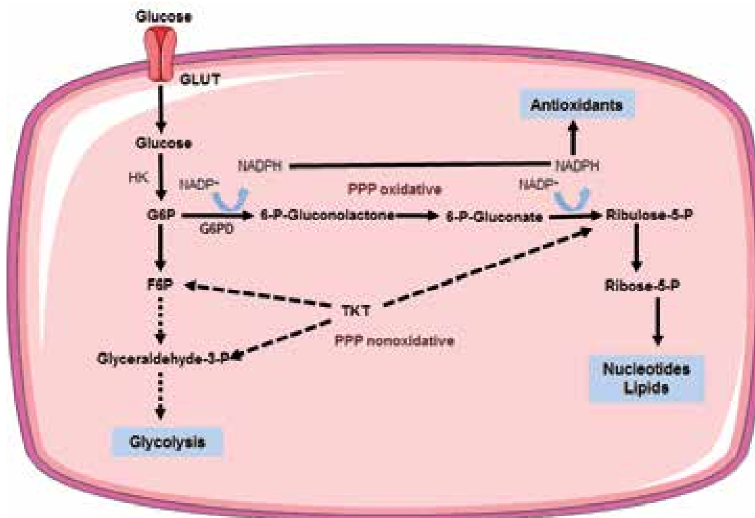


**Figure 4.** Schematic representation of the PPP and its connection with glycolysis. The oxidative branch of the PPP yields NADPH, which can be used for antioxidant defense and biosynthetic reactions. The nonoxidative branch produces ribulose-5-phosphate and glycolytic intermediates. G6PD – glucose-6-phosphate dehydrogenase; TKT – transketolase.

Considering its importance for proliferation, it is not astonishing that increased PPP flux and overexpression of G6PD have been found in several tumors like large B-cell lymphoma or lung adenocarcinoma. It was also shown that it plays a role in promoting malignant cell growth and inducing anchorage-independent growth in NIH3T3 (mouse embryonic fibroblast cell line) cells overexpressing G6PD [40, 41]. These findings suggest G6PD as an oncogene and interesting target for tumor therapy, which is also supported by the fact that the tumor suppressor p53 can bind to G6PD and inhibit its dimerization leading to a decreased G6PD activity, reduced glucose consumption and NADPH production, and, therefore, decreased tumor cell growth [36]. Furthermore, combinations with G6PD inhibitors like DHEA or 6-aminonicotinamide (6AN) with chemotherapeutics like 2-deoxy-D-glucose or oxythiamine, a TKT inhibitor, are shown to increase the inhibition of cancer growth and enhance the radiosensitivity of human gliomas and squamous carcinoma cell lines [42, 43]. Also, many chemotherapeutics like 5-fluoracil or gemcitabine led to the induction of ROS via DNA damage, which could be enhanced by the use of a G6PD inhibitor, and strengthened the importance of the PPP as a target for chemotherapy.

#### *2.3.2. Lipid metabolism*

Not only glucose metabolism is altered in cancer cells but also lipid biosynthesis is enhanced to meet the requirements of fast-proliferating cells. Normal cells obtain lipids via the uptake of free fatty acids (FAs) or low density lipoproteins (LDL) from the bloodstream. New synthesis of fatty acids and cholesterol is restricted to a few specialized tissues like the liver, adipose tissue, or the lactating breast. In cancer cells, however, these restrictions are interrupted and new synthesis of lipids is observed. Lipids are needed for the new synthesis of phospholipid membranes, lipid modified signaling molecules, and as energy storage to survive times of nutrient deprivation. FAs consist of a terminal carboxyl group and a hydrocarbon chain, mostly occurring in even numbers of carbons that can be either saturated or unsaturated. The acetyl groups needed for fatty acid biosynthesis are mostly provided by citrate, which is generated in the TCA cycle. The rate-limiting step of lipid synthesis is the conversion of acetyl-CoA to malonyl-CoA by the acetyl-CoA-carboxylase (ACC), which is then further processed by fatty acid synthase (FAS) to yield saturated and unsaturated fatty acids as shown in Figure 5. Several enzymes in fatty acid biosynthesis have been explored as potential targets for tumor therapy. Especially FAS and ACC are in focus as their inhibition by siRNA or chemical inhibitors led to growth arrest of tumor cells [44].

FAS is a prominent target as inhibition preferentially kills tumor cells and many tumors show increased FAS expression and dependence on *de novo* fatty acid synthesis, whereas nontumor cells rely on exogenous FAs. Inhibition of FAS, for example, leads to apoptosis induction of cells derived from lymph node metastasis of prostate carcinoma LNCaP cells and to an inhibition of HER2 expression in breast cancer cells [45, 46].

ACC is the most regulated enzyme in FA-synthesis. It is positively regulated by citrate and glutamate and inactivated by AMPK. Inhibition of ACC by siRNA or the chemical inhibitor soraphen A induced apoptosis in breast cancer and prostate cancer cells [47, 48]. Another ACC inhibitor, TOFA, showed growth inhibition of ovarian cancer cells and ovarian tumor mouse xenografts [49].

Apart from fatty acids, cholesterol plays a critical role in tumor growth and survival. Cholesterol is either obtained by uptake of LDL from the extracellular environment by the LDL receptor (LDLR) or it is newly synthesized in the mevalonate pathway (Figure 5). The first steps of the mevalonate pathway include the condensation of acetyl-CoA with acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The reduction of HMG-CoA to mevalonate by the HMG-CoA reductase is the rate-limiting step in cholesterol synthesis. Cholesterol is an important component of biological membranes as it modulates fluidity of the lipid bilayer and is part of the detergent-resistant lipid rafts that are membrane parts with high lipid content, which coordinate activation of a variety of signal-transduction pathways. Intermediates of the mevalonate pathway like geranylgeranyl or farnesyl are responsible for isoprenylation of small GTPases like Ras or Rho. Small GTPases play an important role in various cellular events (e.g., intracellular signal transduction, proliferation) and are anchored in the membrane. Accumulation of cholesterol has been associated especially with prostate cancer and an aberrant mevalonate pathway has been linked to several cancers [50].

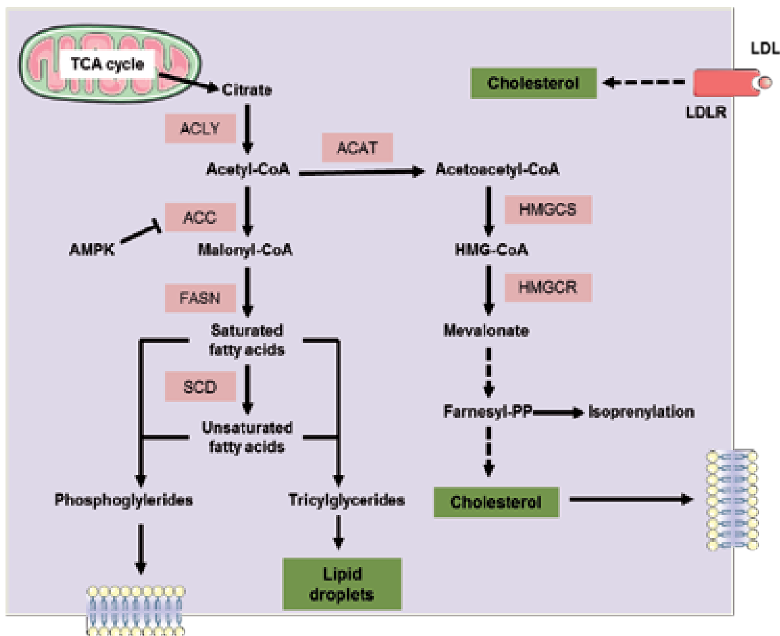


**Figure 5.** Schematic overview of the lipid and cholesterol metabolism. Phosphoglycerides, which are needed for membrane components, and triglycerides, which are stored in lipid droplets, are generated via multiple steps from fatty acids. Cholesterol is produced either by uptake of LDL or by synthesis via the mevalonate pathway and is an important component of the plasma membrane. Intermediates are used for isoprenylation of small GTPases. ACLY – ATP citrate lyase; ACC – acetyl-CoA-carboxylase; FASN – fatty acid synthase; SCD – steaoryl CoA desaturase; ACAT – acetyl-CoA-acetyltransferase, LDLR – low density lipoprotein receptor; HMGCS – 3-hydroxy-3-methylglutaryl-CoA synthase; HMGCR – HMG-CoA reductase.

The HMG-CoA reductase is the target for a group of cholesterol lowering drugs, the statins. Interestingly, there are some reports that statins reduce the risk for some cancers like colorectal cancer or hepatocellular cancer. But other studies found no connections. The effect seems highly dependent on the tumor type. Statins show antiproliferative activities and apoptosis induction in a variety of cancers. The cytostatic properties of statins are mainly due to, on the one hand, inhibition of cholesterol synthesis and, on the other hand, inhibiting the formation of isoprenoids. This could be confirmed by the addition of mevalonate or geranylgeranyl pyrophosphate, which could overcome the antiproliferative effects of statins [51]. Blocking the activity of small GTPases like the oncogene Ras leads to inhibition of pro-survival signaling cascades.

The induction of apoptosis by statins has been widely studied and several mechanisms of action have been revealed. In breast cancer cells, it was shown that apoptosis is induced by the activation of the c-Jun terminal kinase JNK and by downregulation of Bcl-2. In chronic myeloid leukemia, simvastatin induces apoptosis by inhibiting the NFκB pathway [52, 53].

These promising preclinical data lead to several phase I and II studies with the result that success of statins in tumor therapy is very tumor-specific and dependent on how important the mevalonate pathway is for the tumor.

### 2.4. pH regulation

#### *2.4.1. Extra-and intracellular pH*

The intracellular and extracellular pH of cells is one of the major factors that influence molecular processes involved in cell cycle progression and proliferation. Therefore, tumor formation and response of tumors to chemotherapeutics is also highly affected by the environmental acidity. Fast-growing tumors require a complementary vasculature to fulfill the extensive need of nutrients and oxygen. But solid tumors often develop faster than the blood supply, which results in a hypoxic environment. Due to the high glycolytic rate, tumor cells produce increased amounts of $H^+$ (lactate, carbonic acids) and therefore create a hostile microenvironment. The resulting acidic environment is toxic for normal cells and establishes an advantage for growth of tumors, which evolve adaptive mechanisms that allow them to survive [54]. By these adaptive mechanisms, tumor cells transport protons and lactic acid into the extracellular space via acid-base regulators like monocarboxylate transporters and $Na^+/H^+$ exchangers, leading to an acidic microenvironment and to a slightly alkaline cytosol. These pH conditions have shown to be beneficial for metastasis, apoptosis resistance, and increased proliferation. An acidic extracellular space promotes the activation of certain proteases (cathepsins, metalloproteinases) that degrade components of the basement membrane and extracellular matrix (ECM) and, therefore, create the prerequisite for metastasis by enabling tumor cells to get to the bloodstream. Cathepsin family members, especially cathepsin B and K, are overexpressed in metastatic tumors and silencing of these cathepsins was shown to reduce tumor cell invasion [55, 56].

Matrix metalloproteinases (MMP) are also more active at acidic pH. They have been considered as prognostic biomarkers for some metastatic cancers and a number of MMP inhibitors have been developed and tested in clinical trials [57].

The slightly alkaline intracellular pH created by tumor cells was shown to promote proliferation and inhibit apoptosis. Caspases, for example, need an acidic pH to be activated and it was shown in a variety of tumor cells that chemotherapeutics that induce apoptosis lead to an acidification of the cytosol. An alkaline pHi also plays a role in the uptake of chemotherapeutics as the most common drugs are weak bases with intracellular targets that are protonated at lower pH and neutral at higher pH. Therefore, permeation through the plasma membrane and accumulation in the cell is impaired [58].

The importance of pH regulation for tumor cells drove the development of drugs that disrupt tumor pH-regulating systems. $Na^+/H^+$ exchangers like NHE1 are the predominant regulators of pHi. Alkalization of the cytosol triggered by NHE1 is linked to malignant transformation. NHE1 inhibition leads to apoptosis induction of different tumor cell lines and xenograft models and showed a decrease in tumor formation with oncogene-transformed fibroblast lacking NHE1 or KRAS-transformed tumor xenografts. But so far there have not been promising effects for NHE1 inhibitors in monotherapy, especially due to immense toxic side effects [54].

The increased glycolytic flux of tumor cells requires a system to export lactic acid from the cell, which is done by monocarboxylate transporters (MCTs). Among the 14 family members, MCT1

and 4 are specialized for the cotransport of lactate and $H^+$, and a high expression of especially MCT4 was found in rapidly growing tumors such as triple-negative breast cancer [59]. Owing to their pivotal role in pHi regulation by securing a slight alkaline cytosolic pH despite high production of lactate, there was ongoing research for the development of small molecule inhibitors. And indeed, MCT inhibition impaired glioblastoma cell proliferation, migration, and survival [60]. Treatment with metformin could sensitize tumor cells to MCT inhibition, which indicates the possibility that combination with other metabolic or pH regulating drugs could target tumors dependent on glycolysis.

#### *2.4.2. V-ATPase*

The vacuolar $H^+$-ATPase (V-ATPase) is a multisubunit enzyme, which is located in the membranes of almost all eukaryotes. It is responsible for acidifying intracellular organelles like endosomes, lysosomes, golgi-derived vesicles, or secretory vesicles and is therefore responsible for the pH homeostasis in the cell. Inhibition of the V-ATPase leads to apoptosis induction and inhibition of migration in a variety of tumor cells and has therefore become an interesting target in tumor therapy [61, 62]. As reported above, regulation of pH is important for tumor cell survival and metastasis as an acidic extracellular microenvironment facilitates metastasis of tumor cells by activating proteases like MMPs. The V-ATPase is expressed on the plasma membrane of metastatic and chemoresistant tumor cells and pumps protons across the membrane creating an acidic microenvironment [63].

Apart from regulating pH we could show that V-ATPase inhibition leads to an increased transcription of genes involved in glycolysis, fatty acid, and cholesterol synthesis. Furthermore, V-ATPase inhibition leads to an increased glucose consumption and especially a strong induction of the hypoxia-inducible factor-1$\alpha$ (HIF1$\alpha$) occurs [64]. As described above, HIF1$\alpha$ is the major inducer of glycolysis and metabolic stress and induction by the V-ATPase inhibitor archazolid strongly indicates that archazolid leads to changes in the tumor metabolism, which could be exploited for cancer therapy.

## 3. Conclusion

Although in recent years much work has been done in understanding the regulation of tumor metabolism and the discovery of drugs that specifically target tumor-related pathways, the complex interplay between oncogenic signaling pathways and tumor metabolism demands further research. Metabolic reprogramming may render cancer cells highly dependent on specific enzymes or processes that could be exploited for cancer therapy and induce apoptosis. But the search for relevant targets may be complicated due to the high diversity of tumor metabolism and the possibility of compensatory mechanisms. Another challenge is to identify those metabolic pathways that are essential targets and understanding the mechanism of apoptosis induction is inevitable in tumor therapy. It is important to understand that there is not one single tumor-specific metabolism but several programs that differ from tumor to tumor and are adjusted to the special requirements of different tumors in different tissues. Therefore,

targeting different enzymes in glycolysis might be beneficial in some tumors but may have no effect in others that still have the ability to use oxidative phosphorylation for energy production. Also, normal cells need to produce energy, nucleotides, or other metabolites, as well and it is important to investigate why there exists a therapeutic window that does not harm normal cells. The complexity of the tumor metabolism furthermore demands *in vivo* models to study the effect of drugs on a whole organism.

Only by further understanding the regulation of tumor metabolism, we will be able to translate this knowledge into the discovery of drugs that will lead to tumor growth inhibition and a better clinical outcome of cancer patients.

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Chapter 17

# Modulation of Host Programmed Cell Death Pathways by the Intracellular Protozoan Parasite, *Toxoplasma gondii* — Implications for Maintenance of Chronic Infection and Potential Therapeutic Applications

Sandra K. Halonen

Additional information is available at the end of the chapter

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**Abstract**

Programmed cell death (PCD) pathways are genetically programmed mechanisms that can trigger the cell to die or commit "cell suicide". There are three major forms of programmed cell death that are now recognized: apoptosis (type I), autophagy (type II) and necrotic cell death or necroptosis (type III). While these cell death processes were once thought to occupy discrete cell states, evidence suggests that apoptosis, autophagy and necrosis are often regulated by similar pathways and share initiator and effector molecules and some subcellular compartments indicating that crosstalk exists between these three main forms of cell death pathways, resulting in a balanced interplay by which the cell decides its fate. PCD pathways have important roles in many cellular processes such as development and oncogenic transformation, but PCD pathways also play important roles in host defense and elimination of pathogens. *Toxoplasma gondii* is a microbial pathogen for which programmed cell death pathways are a key part of the host defense. *T. gondii* is an obligate intracellular protozoan parasite that infects approximately one-third of the world's population. In most immunocompetant individuals, the chronic infection is asymptomatic due to an effective immune response that eliminates active parasite replication. The parasite has evolved immune evasion strategies that enable it to survive and persist long enough in the host however to establish a chronic infection in which the cyst stage persists within neurons in the brain and skeletal muscle in the periphery. *T. gondii* has evolved multiple mechanisms to resist killing by apoptotic, autophagic and necrotic cell death pathways, and the parasite's manipulation of host PCD pathways plays a crucial role in host–parasite interactions and maintenance of the chronic infection. While most individuals chronically infected with *T. gondii* are asymptomatic, severe disease can occur in immunocompromised individuals where the infection reactivates from the brain causing severe necrotizing encephalitis, and increasing evidence indicates chronic cerebral toxoplasmosis in some individuals may lead to neuropsychiatric disorders such as schizophrenia and suicidal behavior. This review will focus on the role of PCD pathways in host defense of *T. gondii* and the parasite manipulation of these PCD pathways. A bet-

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ter understanding of the molecular components underlying the PCD pathways and the parasite manipulation of these pathways may yield new therapeutic targets for treatment of clinical sequelae of cerebral toxoplasmosis.

**Keywords:** Apoptosis, autophagy, necroptosis, necrosis, toxoplasmosis

## 1. Introduction

### Overview of Programmed Cell Death (PCD) pathways and applications to *Toxoplasma gondii* infection

Programmed cell death (PCD) pathways are genetically programmed mechanisms that can trigger the cell to die or commit "cell suicide". There are three major forms of programmed cell death which are now recognized: apoptosis (type I), autophagic cell death (type II) and necrotic cell death or necroptosis (type III) [1–4]. Apoptosis, the best-characterized and dominant form of PCD, is a controlled physiological process of cellular self-destruction that removes dead cells without damage to the host. Apoptosis is critically important during development and morphogenesis and eliminates damaged cells such as cancerous cells or infected cells that may interfere with normal function. Necrosis, conversely, is a cell death process that is observed in response to severe stresses such that occurs after physical injury or prolonged ischemia and has been long thought to be an unregulated process. However, a programmed form of necrotic death, called necroptosis, is now recognized that can occur during physical traumas, in neurodegeneration, in cell death due to ischemia or infection and that, in contrast to unordered necrosis, has dedicated molecular pathways controlling necrotic cell death. Autophagy is predominantly a strategy for survival and not death, serving as a housekeeping mechanism of normal turnover of long-lived proteins and whole organelles and crucial to maintenance of healthy cells. However, autophagy can promote cell death during normal development and excessive autophagy, stimulated in times of stress such as nutrient deprivation and some diseases, can lead to what is now recognized as autophagic cell death [5–7]. While these cell death processes were once thought to occupy discrete cell states, evidence suggests apoptosis, autophagy and necroptosis are often regulated by similar pathways, share initiator and effector molecules and some subcellular compartments indicating a complex crosstalk exists between these three main forms of cell death pathways, resulting in a balanced interplay by which the cell decides its fate [8].

PCD pathways are also an important part of host defense against intracellular pathogens. The intracellular protozoal pathogen, *Toxoplasma gondii,* is a common infection that causes a chronic infection of central nervous system that persists for the lifetime of the individual. PCD pathways are a key part of the host defense against *T. gondii*, but the parasite has evolved multiple mechanisms to resist killing by host cell death pathways. Parasite manipulations of host cell death pathways are essential components leading to successful establishment of infection and for maintenance of the chronic infection in the host. In this review, the molecular components and signaling pathways of each of the three main types of PCD pathways will be

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discussed with the role of these PCDs in the pathogenesis of the intracellular pathogen, *T. gondii*, and potential therapeutic targets for toxoplasmosis and other diseases, the focus of this review.

## 2. Apoptosis, necroptosis and autophagic cell death pathways: Morphological features and molecular components

Apoptosis, necroptosis and autophagic cell death pathways operate via distinct genetically programmed molecular mechanisms containing dedicated molecular components and leading to characteristic morphological features (Figure 1). Apoptosis operates via a caspase-dependent mechanism while necroptosis and autophagic cell death are caspase-independent processes, with autophagic cell death being cathepsin-dependent and necroptosis mediated via the receptor interacting protein kinase 3 (RIP3) and its substrate, the mixed lineage kinase-like domain protein, MLKL [8]. Apoptosis results in mitochondrial membrane permeabilization, chromatin condensation and DNA fragmentation, resulting in cells become smaller (pknotic) and membrane blebbing into apoptotic bodies. Phagocytes, such as macrophages, subsequently engulf apoptotic cells and hence apoptosis is considered a noninflammatory form of programmed cell death. Necrotic cell death, in contrast to apoptosis, is marked by swelling of ER and mitochondrial organelles, increase in cell size, rapid rupture of the plasma membrane resulting in lysis of the cell and release of danger-associated molecular patterns (DAMPs) which stimulate inflammation. While necrosis has traditionally been considered to be "pathological cell death", necroptosis is now recognized to occur via a regulated mechanism that is inhibited by caspases [9, 10]. Autophagy is the process by which cells recycle cellular constituents and involves engulfment of cytoplasmic material and intracellular organelles within double-membrane vesicles called autophagosomes that fuse with lysosomes to make autophagolysosomes that degrade the cellular cargo via cathepsins located in the autolysosomes. The molecular components and signaling pathways of each cell death processes are reviewed below, followed by the current understanding of the crosstalk that exists between these PCD pathways.

### 2.1. Apoptosis

The term apoptosis was first used in 1972 by Kerr, Wyllie and Currie [11] to describe a form of cell death morphologically distinct from necrosis. Apoptosis has since been well studied and is now understood to be a regulated energy-dependent process mediated via cysteine-dependent aspartate-directed enzymes called caspases [3]. Apoptosis is a homeostatic process that balances cell numbers and plays a crucial role in several physiological processes including embryogenesis to shape morphological structures such as fingers, in the establishment of functional synaptic connections in the nervous system, in development of the immune response to remove self-reactive lymphocytes and at the termination of the immune response to remove antigen-specific lymphocytes. Apoptosis is also used to rid the body of cells in various pathological conditions such as removal of cancerous cells, infected cells or cells damaged by noxious agents [12].

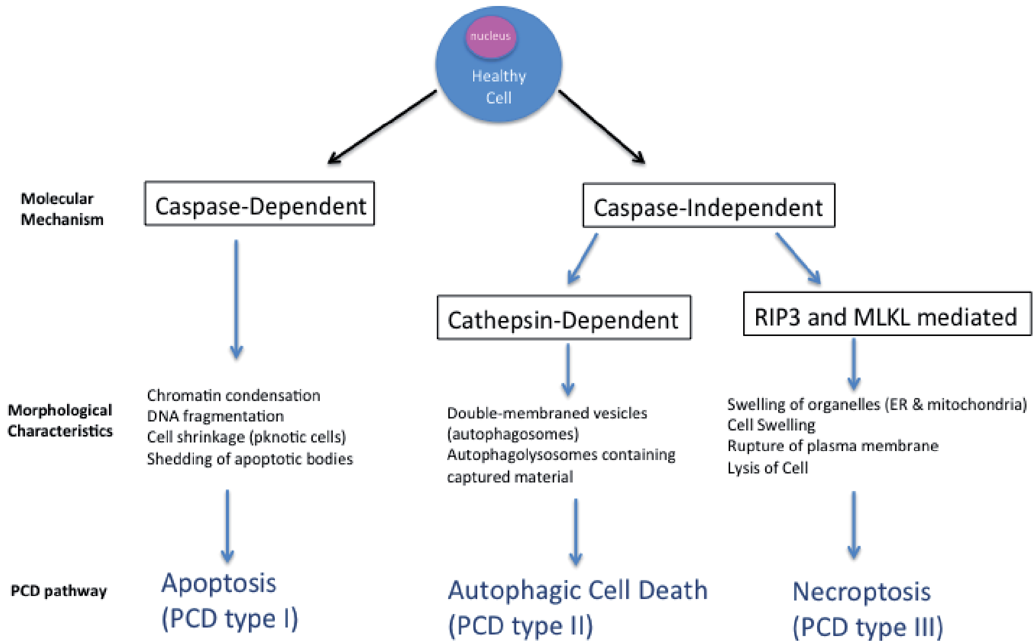


**Figure 1. Comparison of three types of Programmed Cell (PCD) Pathways.** The molecular mechanisms and morphological characteristics of Apoptosis, Autophagic Cell Death and Necroptosis cell death pathways, so-called PCD types I, II and III, respectively, are indicated. RIP3 = receptor-interacting protein kinase 3; MLKL = mixed lineage kinase-like domain protein

### *2.1.1. Signaling pathways and molecular components of apoptosis*

Apoptosis is mediated via caspases which are present in the cytoplasm as pro-enzymes and when activated initiate a proteolytic cascade that results in apoptosis of the cell. There are three activation pathways that can initiate apoptosis: the extrinsic (death receptor) pathway, the intrinsic (mitochondrial) pathway and the granzyme pathway (Figure 2). There are about 14 known caspases which are divided into initiator and effector caspases. Initiator caspases, caspases 8, 9 and 10, are triggered by either the extrinsic, intrinsic or granzyme pathway, respectively. All three pathways converge on caspase 3, which activates effector caspases 6 and 7 that lead to apoptosis. A major target of effector caspases is poly-ADP-ribose polymerase (PARP) which is involved in DNA repair, cell survival, proliferation and differentiation.

The extrinsic pathway is initiated by binding of cell membrane receptors of Fas (CD95) or tumor necrosis factor (TNF) family receptor (TNFR), which after binding its relevant ligand of FasL or TNF$\alpha$, respectively, trimerizes and attracts the docking proteins Fas-associated death domain (FADD) and TNF receptor-associated death domain (TRADD), respectively. FADD

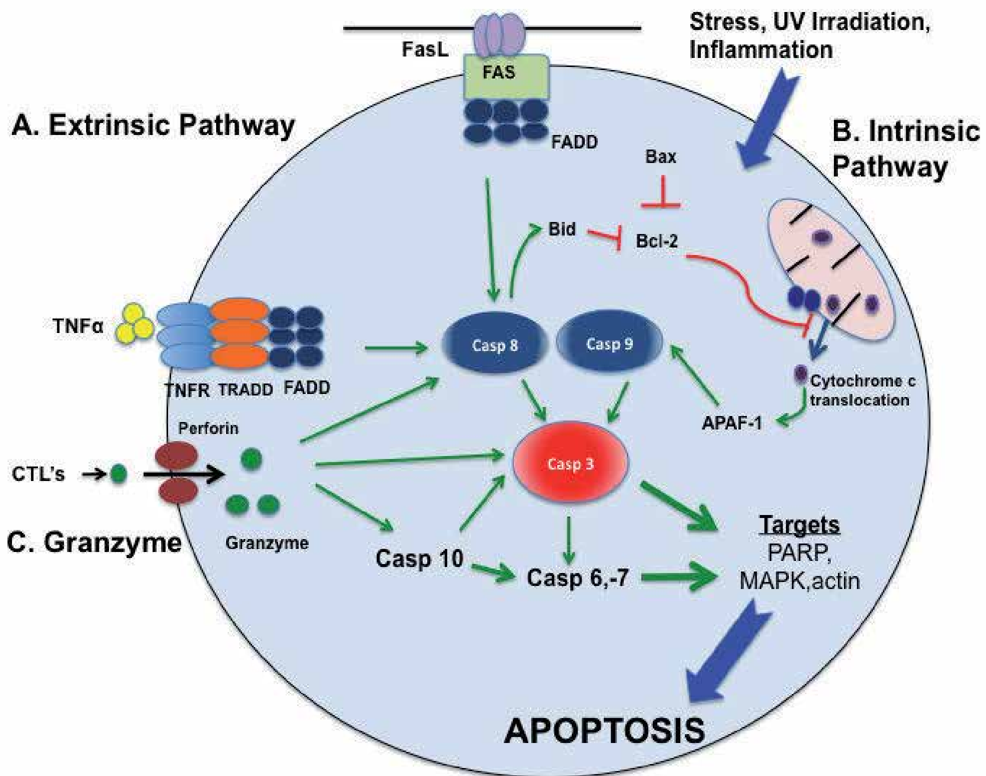


**Figure 2. Signaling Pathways of Apoptosis.** Apoptosis is induced through either the extrinsic pathway (A), intrinsic pathway (B) or the granzyme pathway (C). Activation of initiator caspases 8 and 9 (Casp 8,-Casp9), leads to apoptotic cell death through activation of Caspase 3 (Casp3) and subsequent activation of effector caspases 6 and 7 (Casp 6-Casp7). The extrinsic pathway (A) is initiated via extracellular molecules, FasL and TNFα, which bind to TNFR family members, Fas and TNFR respectively which activate Casp8 (green lines). The intrinsic pathway (B) is initiated by stress, UV irradiation and inflammation which act on the mitochondria through the pro-apoptotic Bcl-2 family members, such as Bax, resulting in the blockage of the anti-apoptotic activity of Bcl-2 (red lines). As a result, Cytochrome c is released into the cytoplasm and activates Casp9 through APAF-1 (green lines). Casp8 may also trigger the intrinsic pathway through activation of Bid, which inhibits the anti-apoptotic activity of Bcl-2. The granzyme pathway (C) is activated via cytotoxic T cells (CTLs) that introduce granzyme molecules into the target cells via secretion of perforin, which through a multimerization process forms a pore in the cell membrane allowing granzyme into the target cell. Granzymes cleave multiple caspases, including Caspase 10.

contains two death effector domains that can bind and cleave caspase 8. TRADD lacks death effector domains but binds FADD that then cleaves caspase 8. Activated caspase 8 then activates caspase 3. The intrinsic pathway is activated via changes in the mitochondria membrane potential which can be triggered via stress, toxic reagents, UV irradiation and inflammation. Changes in mitochondria membrane result in translocation of cytochrome c from the inner mitochondrial membrane into the cytoplasm. Cytochrome c binds to apoptosis protease activating factor 1 (APAF-1) which in the presence of ATP cleaves caspase 9. The intrinsic pathway is regulated by Bcl-2 which localizes to the outer mitochondria membrane. Bcl-2 members (Bcl-2 and Bcl-x) are anti-apoptotic while others such as Bax and Bad are pro-

apoptotic. The granzyme activation pathway is stimulated by cytotoxic T lymphocytes (CTLs) or NK cell secretion of perforin, which creates pores in the target cell membrane allowing granzymes into the cytoplasm activating caspase 10 which can then activate caspase 3.

*2.1.2. Apoptosis in physiological and pathological conditions and potential therapeutic application*

Apoptosis is a tightly regulated process and is rarely observed in healthy animals because phagocytes rapidly remove apoptotic cells. Abnormalities in cell death regulation can be a significant component of diseases such as cancer, AIDS and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis. Some of these conditions are characterized by insufficient apoptosis and others excessive apoptosis. Excessive apoptosis, for example, is a feature of neurodegenerative diseases while resistance to apoptosis characterizes many cancers. Understanding the mechanisms of apoptosis at the molecular level may provide therapeutic interventions in these diverse disease processes.

*2.1.2.1. Autophagic cell death*

Autophagy, which means literally to "eat oneself", is a physiological response to stress such as starvation and is considered the major cellular mechanism for generating the needed metabolic sources of energy and metabolites during times of nutrient deprivation. Autophagy can also be used to remove damaged or unwanted organelles, such as mitochondria and long-lived proteins. Autophagy is a homeostatic mechanism that is predominantly a cytoprotective process [7, 13]. Other substrates are also now recognized as potential cargo for autophagy including lipids, nucleic acids, reticulocytes and intracellular pathogens and viruses. The concept of autophagic cell death has been a matter of debate, but it is now recognized as a type of programmed cell death mechanism that can lead to both apoptotic and necrotic cell death in certain circumstances such as extreme stress conditions [14, 15]. Autophagic cell death or type II programmed cell death is now used to refer to cell death process distinct from apoptosis that is caspase-independent and occurs with accumulation of double-membrane organelles called autophagosomes.

*2.1.3. Signaling pathways and molecular components of autophagy*

Autophagy delivers cytosolic materials to the interior of the lysosomes for degradation via a process involving the "*de novo*" formation of cytoplasmic double-membrane vacuoles called autophagosomes which then fuse with lysosomes forming an autolysosome that degrades the cellular cargo (Figure 3). Autophagy is regulated by a genetic program with a number of autophagy-related genes (Atg) whose gene products (ATG proteins) regulate distinct steps in autophagy. The autophagic pathway proceeds through the following defined steps: (i) initiation phase involves the formation of an isolation membrane or phagophore, (ii) elongation of the phagophore, (iii) maturation of an autophagosome with accumulation of cytosolic cargo, (iv) fusion of the mature autophagosome with the lysosome and (v) degradation of the contents via lysosomal proteases (i.e., cathepsins) in the autolysosome. The initiation phase involves formation of an initiation complex comprised of the class III PI3 kinase, VSP34, which

converts phosphatidylinositol to phosphatylinositol-3-phosphate (PI3P). The activity of VSP34, which binds to beclin 1, requires the activity of VSP15, the regulator beclin 1 and ATG14L (Figure 3a). Elongation of the autophagosome membrane requires action of two ubiquitin-like conjugation systems, the Atg5–Atg12 conjugation system and the microtubule-associated protein-1 light chain (LC3). The phosphatidylethanolamine-conjugated form of LC3, LC3-PE (also called LC-II) is generated by ATG4-dependent proteolytic cleavage of LC3 and the action of the E1 ligase, ATG7, the E2 ligase, ATG3 and the E3 ligase complex, ATG12/ATG5/ATG16L. LC3-PE stably associates with the autophagosome and is commonly used as a marker for autophagosomes.

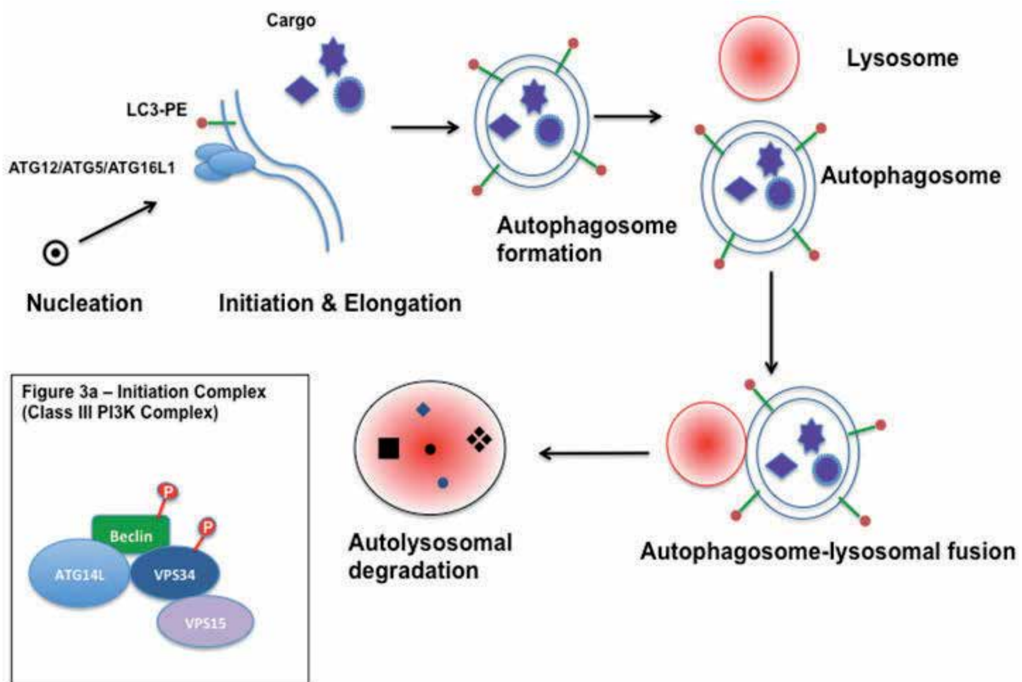


**Figure 3. Autophagy Pathway**. Autophagy is a membrane-dependent pathway that involves a series of defined steps beginning with a nucleation step which then leads to: 1). Initiation of the isolation membrane or phagophore, 2). Formation of the autophagosome around cellular cargo, 3). Fusion of autophagosome with lysosomes, and 4). Degradation of cargo by lysosomal proteases in autolysosomes. Formation of the initiation complex (also called Class III PI3K Complex) as shown in Figure 3a, consists of Beclin-1, ATG14L, VPS34 and VPS15. Activation of the initiation complex requires disruption of binding of Bcl-2 to Beclin-1. The Class III PI3K complex generates PI3P at the site of nucleation of the isolation membrane, which leads to binding of PI3P-binding proteins and the subsequent recruitment of proteins involved in the 'elongation reaction' of the isolation membrane. These proteins contribute to membrane expansion resulting in the formation of a double-membrane structure, the autophagosome that surrounds cellular cargo. The phosphatidylethanolamine-conjugated form of the LC3 (LC3-PE) is generated by the ATG4 dependent proteolytic cleavage of LC3 and the action of E1 ligase, E2 ligase and the E3 ligase ATG12/ATG5/ATG16L. LC3-PE stably associates with the autophagosome and is a marker of autophagosomes and therefore LC3 is commonly used as a marker of autophagy pathway.

While the autophagy pathway is primarily a homeostatic process promoting cell survival, increased autophagosomal formation can occur coincidentally with cell dying indicating autophagy may be involved in regulated cell death pathways, most notably apoptosis [7, 13]. For example, in apoptosis-comprised cells, cells die via a caspase-independent mechanism characterized by autophagosome accumulation, implicating a role for autophagy in the cell death process. Autophagy and apoptosis may also be regulated coordinately as anti-apoptotic proteins that downregulate apoptosis can also downregulate autophagy. For example, members of the Bcl-2 family can bind to beclin-1, thus inhibiting the formation of initiation complex that is necessary to stimulate autophagy. Other studies indicate once apoptosis is activated, apoptosis effector molecules may suppress autophagy as beclin-1 is cleaved and inactivated by caspases. Finally, studies also indicate autophagy proteins may play a dual role in regulation of apoptosis and autophagy. For example, Atg5 may affect the extrinsic apoptotic pathway through interaction with FADD proteins, while Atg12 is an effector of intrinsic apoptotic pathway with both Atg5 and Atg12 acting as pro-apoptotic regulators.

Autophagy pathway has also been implicated in necrosis and necroptosis cell death. Evidence indicates autophagy may be able to act as an inhibitor of necrosis/necroptosis by preserving cellular functions, removing toxic products and maintaining cellular energy. For example, knockdown studies of components of autophagy pathways, such as beclin-1, have found cytotoxicity is exacerbated suggesting that autophagy has a cytoprotective role. Autophagy may be able to act as a buffer to metabolic stress, providing a mechanism to generate ATP to maintain metabolic viability and thus prevent necrotic cell death. Conversely, studies using a specific inhibitor of necroptosis, necrostatin-1, found both necroptosis and autophagy were inhibited, indicating autophagy may be induced by necrosis. Molecular components underlying the relationship between autophagy and necroptosis/necrotic cell death at this point are poorly understood.

*2.1.4. Physiological and pathological roles of autophagic cell death and therapeutic implications*

Thus, while autophagy is now recognized to play a role in apoptotic and necrotic cell death programs, this process is complex and incompletely understood. However, increasing evidence indicates autophagic cell death may be necessary for cell death in certain circumstances. It has been suggested autophagic cell death represents a failed adaptive mechanism that may have prevented death under milder conditions. Understanding of the relationships of autophagic cell death with other programmed cell death modalities will require further study. The study of autophagy in disease is an emerging area of research. Autophagy pathway and/or autophagic cell death pathways which result in failure to remove damaged organelles and/or damaged cells are now thought to contribute to various diseases such as cancer, neurodegenerative diseases such as Parkinson's disease, aging and inflammation. Elucidation of the influence of autophagy with other cell death programs will be essential to the development of therapeutics targeting autophagy for treatment of these various diseases. In the context of infectious disease, autophagy also is now recognized to play an important role in intracel-

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lular pathogen defense, and it has been suggested that intracellular pathogen load could be a factor that disrupts the balance between cell survival and cell death [16].

### 2.2. Necroptosis

Pathologists first used the term "necrosis" in the early 19th century to describe tissue destruction. Necrosis is characterized by organelle swelling and cell lysis, releasing the cellular content of cells and resulting in inflammation. The first indication that necrosis could occur in a regulated manner arose from observation that tumor necrosis factor (TNF$\alpha$) could trigger apoptosis and necrotic forms of cell death. Caspase inhibition strongly exacerbates necrotic cell death, indicating caspase activity negatively regulates necrosis. This accumulating evidence led to the concept of "programmed necrosis" and the coining of the term necroptosis in 2005 to designate regulated necrotic cell death [4, 9]. Receptor-interacting protein kinase 3 (RIP3) and its substrate, the mixed lineage kinase-like domain protein (MLKL), are now recognized as the key activation steps and the molecular hallmarks of regulated necrotic cell death or necroptosis.

#### *2.2.1. Signaling and molecular components of necroptosis*

Programmed necrotic cell death can be activated when cells are stimulated by ligation of death receptors including CD95 (also known as FAS), TNF receptors (TNFRs) and TNF-related apoptosis-inducing ligand (TRAIL). Ligation of cell death receptors leads to the formation of complex 1, comprised of TNFR-associated death domain protein (TRADD), TNFR-associated factor 2 (TRAF2), receptor-interacting kinase 1(RIP1) and cellular inhibitors of apoptosis (CIAPs) (Figure 4). Upon the release of second mitochondria-derived activator of caspases (Smac) from the mitochondria, RIP1 is de-ubiquinated and dissociates from the death receptor and induces the formation of FADD-RIP1-caspase 8 pro-apoptosis complex II. Conversion of complex 1 to complex II indicates a change in cell fate from pro-survival to cell death. When caspase 8 is inhibited, RIP1 and RIP3 form the necrotic death domain complex, the necrosome, and their kinase activities become activated. RIP3 recruits it substrate monomeric MLKL from the cytoplasm and phosphorylates it. The phosphorylation destabilizes MLKL and drives its oligomerization which enables it to bind to phosphatidylinositol phosphate lipids (PIPs) and cariolipin (CL). Different PIPs and CLs orchestrate the translocation of MLKL to different membrane compartments including the mitochondria, endoplasmic reticulum, the Golgi, lysosomes and plasma membranes. In addition to activation via death receptors, it is also now clear that necroptosis can also be initiated by pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs) and cytokines via activation of RIP3 through distinct upstream mechanisms.

#### *2.2.2. Physiological and pathological roles of necroptosis and potential therapeutic applications*

Programmed necrosis has been found to be important in host antiviral responses and a variety of tissue-damage related diseases such as acute pancreatitis, ischemic reperfusion injury, retinal detachment, atherosclerosis, neuronal loss in Gaucher's disease, amongst others. It has

been a paradigm that apoptotic cell death is anti-inflammatory with dead cells cleared by phagocytes while necrotic cell death leads to inflammation and tissue damage. Necrotic cells, however, can be internalized by macrophages although via macropinocytosis and thus in a manner distinct from phagocytosis of apoptotic cells, but they are nonetheless efficiently cleared by professional and nonprofessional phagocytes and hence rarely found in tissues. Large numbers of dying cells such as may occur in excessive tissue injury, autoimmune diseases such as systemic lupus erythematosus or infectious disease may lead to the defective clearance of necrotic cells and contribute to the persistent inflammation and tissue damage. However, smaller number of cells dying via necrosis or necroptosis may not necessarily lead to massive tissue damage and hence controlled necrotic cell death may not necessarily lead to massive tissue damage commonly associated with necrotic cell death.

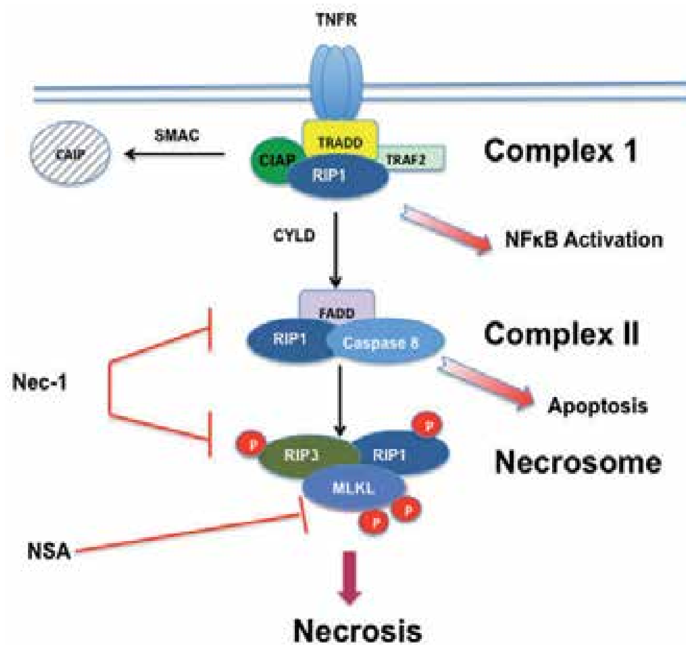


**Figure 4. Necroptosis Signaling Pathway**. Ligation of tumor-necrosis factor 1 (TNFR1) leads to the formation of Complex 1 that is comprised of TNFR-1, TFNR1-associated death domain (TRADD), TNFR-associated factor 2 (TRAF-2), Receptor-interacting kinase 1 (RIP1) and cellular inhibitors of apoptosis (CIAPs). RIP1 is initially modified by ubiquitin chains within Complex I after recruitment to the membrane and serves as the signaling platform for NF-κB activation. Upon release of the second mitochondria-derived activation of caspases (Smac) from the mitochondria, CIAPs are auto-degraded. RIP1 is subsequently de-ubiquinated by CYLD and dissociates from the death receptor, and associates with FADD-RIP1-Caspase8 forming Complex II, which is pro-apoptotic. Within Complex II, Caspase 8 activates Caspase 3/7 to induce apoptosis and blocks necrosis by cleaving and inactivating RIP1 and RIP3. When apoptosis is inhibited, as for example by Z-VAD, RIP1 and RIP3 form the necrotic death complex, the necrosome, and their kinase activities become activated. RIP3 recruits the mixed lineage kinase domain-like protein, MLKL, and phosphorylates MLKL, causing its activation. Activated MLKL then associates with membranes such as the plasma membrane, mitochondrial members and others, causing lysis of these membranes and death of the cell. Inhibitors of necroptosis include, Necrostatin 1 (Nec1) which inhibits RIP1 dependent necrosis by inhibiting the kinase activity of RIP1 to prevent necrosome formation and Necrosulfonamide (NSA), which inhibits MLKL-mediated necrosis.

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Knockout of either RIP3 or MLKL genes in mice has been used to assess the physiological and pathological roles of programmed necrosis. These types of studies have confirmed that necroptosis is important in host antiviral responses and in some tissue-damage related diseases. Several inhibitors have been identified that can inhibit necroptosis. Necrostatins, for example, are small molecules that inhibit RIP1 kinase activity and thus inhibit necroptosis (Figure 4). RIP1 also contributes to other processes such as apoptosis and NK-kB activation, however, indicating other processes may also be affected by Nec-1. Targeting RIP3 or MLKL offer more specific inhibitors for study or treatment of necroptosis. A chemical inhibitor of MLKL called necrosulfonamide (NSA), for example, blocks necrosis by preventing oligomerization of MLKL and thus is a specific inhibitor of necroptosis (Figure 4). Use of these specific inhibitors of necroptosis will be essential to elucidate the role of necroptosis in cell death. As the role of programmed necrotic death is now known to occur in several human diseases, development of specific inhibitors of neroptosis could also have therapeutic uses.

### 2.3. Crosstalk between apoptosis, necroptosis and autophagy PCD pathways

As molecular components and regulation of each pathway has become better understood, it has become apparent that significant crosstalk exists between the three major pathways of programmed cell death, the focus of which has been the topic of several recent reviews [3, 8, 10, 13]. Extensive crosstalk exists between apoptosis and autophagy as discussed above in the section on autophagic cell death. The stimuli for apoptosis and autophagy are often the same and evidence indicates instances where they can cooperate, antagonize or assist each other. Caspases, Beclin-1 and/or the TOR kinase pathway have all been implicated in participating in the complex crosstalk between apoptosis and autophagy. Crosstalk between apoptosis and necrosis also appears to exist as receptors that stimulate apoptosis can also stimulate necroptosis. Energy is thought to play a central role in determining the interplay between these two pathways where instances of high ATP levels would enable a cell to undergo apoptosis while low ATP levels would favor necrosis. Other factors such as p53, Bcl-2 proteins and PARP1 are also suggested to play a role. Evidence also suggests a complex interplay between autophagy and necroptosis. Autophagy and necrosis can be activated in parallel or sequentially and can have either opposite or the same effects. Autophagy, for example, can protect against some types of necroptotic cell death with autophagy serving as the last resort before cell death via necrosis. The molecular basis for interlinked processes in autophagy and necroptosis and the impact of autophagy on necroptosis and other cell death pathways [13], however, remain unclear. While the molecular details are not fully understood, it is clear that a complex crosstalk and molecular interplay between apoptosis, autophagy and necroptosis occur and determine the ultimate fate of the cell to survive or die in a given situation or under a given stress signal. A better understanding of the mechanisms of apoptosis, autophagic cell death and necroptosis at the molecular level and the crosstalk between these cell death pathways would provide a deeper insight into different disease processes and may provide novel therapeutic strategies.

## 3. *Toxoplasma gondii:* Manipulation of programmed cell death pathways and impact on host–parasite interactions and maintenance of chronic infection

PCD can be important host cell defense mechanisms to eliminate intracellular pathogens. *Toxoplasma gondii* is an obligate intracellular protozoan parasite that causes a chronic infection with approximately one-third of the world's population chronically infected, making *T. gondii* one of the most prevalent human parasitic infections worldwide [17–19]. In humans, infection with *T. gondii* is characterized by rapid parasite replication and dissemination throughout the body with the parasite capable of infecting all types of nucleated cells. The acute infection is followed by a chronic infection that lasts for the lifetime of the individual with the parasite harbored within neurons in the brain and muscle tissue in intracellular cysts. In most immunocompetant individuals, the infection is asymptomatic due to an effective immune response that eliminates active parasite replication. In immunocompromised individuals such as AIDS patients that are chronically infected, the parasite reactivates from cysts in the brain leading to severe and potentially fatal encephalitis. The parasite can also be transmitted transplacentally and can cause severe neurological complications to the fetus including mental retardation, hydrocephaly and chorioretinitis which can reactivate through the first two decades of life potentially leading to blindness. Finally, increasing evidence indicates that in some immunocompetant individuals, chronic infection with *T. gondii* may lead to development of serious psychiatric disorders such as schizophrenia or suicidal tendencies [20–22].

While the host mounts an effective immune response against *T. gondii*, the parasite has evolved immune evasion strategies that enable it to survive and persist long enough in the host to establish the cyst stage of the infection that can persist for the lifetime of the host. PCD pathways are a key part of the host defense against *T. gondii*, but the parasite has evolved multiple mechanisms to manipulate these pathways. For example, the parasite induces apoptosis in selected immune cell lineages, thus suppressing the host immune response and helping to establish infection but the parasite also has multiple mechanisms to resist killing by apoptotic and, to lesser degree, autophagic and necrotic cell death pathways in different host cell types. Thus, parasite manipulations of host cell death pathways appear to be an essential component to the successful establishment of infection and maintenance of the chronic infection. A brief summary of the mechanisms by which the parasite manipulates these cell death pathways is reviewed below followed by a discussion of the potential therapeutic interventions.

### 3.1. Apoptosis

*T. gondii* has the ability to promote apoptosis of immune effector cells early after infection in selected cell types. In murine models of toxoplasmosis, apoptosis of CD4+, CD8+ T cells, B cells, NK cells and granulocytes is observed in the spleen early after infection [23]. Apoptosis

in Peyer's patch T cells in perorally infected mice has also been observed [24]. Acute infection of *Toxoplasma* in both mice and humans induces a state of transient immunosuppression as determined by decreased antibody and T lymphocyte responses. Triggering of apoptosis of T cells and other immune effector cells by *T. gondii* may be one factor by which the parasite restricts the immune response, thus allowing the establishment of infection. Induction of apoptosis of immune effector cells may be somewhat parasite strain-dependent as, in murine models, high levels of apoptosis in T cells was induced when mice were infected with virulent RH strain while this phenomenon was not seen in mice infected with avirulent strains.

Inhibition of apoptosis by infection with *T. gondii* has been found to occur in a wide range of cell types including macrophages and a wide variety of non-immune effector cells such as fibroblasts, endothelial cells, muscle cells and astrocytes [23, 25, 26]. Infected host cells are resistant to induction of apoptosis to a wide range of stimuli including CTL-mediated cytotoxicity, irradiation, growth factor withdrawal, TNF$\alpha$ and several toxic reagents. Blockage of apoptosis in host cells establishes an anti-apoptotic condition of the host cell and favors parasite persistence. Neighboring uninfected cells are also rendered resistant to apoptosis. Inhibition of host cell apoptosis of non-immune effector cells likely ensures the host cell stays alive long enough to facilitate intracellular replication of the parasite in the tissues while suppression of apoptosis of uninfected neighboring cell may help create a microenvironment in different tissues in which the parasite can persist and replicate.

While blockage of apoptosis is known to occur in a wide variety of host cells, modulation of apoptosis in cells of the central nervous system (CNS) is less well studied and results are ambiguous. *T. gondii* can infect neurons, astrocytes and microglia supporting growth of the rapidly replicating tachyzoite form while bradyzoite stage and cysts develop only in neurons and astrocytes. In murine astrocytes, infection blocks apoptosis beginning at 6–24 h after infection, allowing time for the parasite to replicate, egress and initiate infection of a new host cell [26]. Thus, inhibition of apoptosis in astrocyte host cells may allow an increase in parasite numbers in the brain and help establish the chronic infection in the brain. Conversely, several studies indicate the parasite induced apoptosis in neurons. For example, tachyzoites induced apoptosis in mouse brain cells in adult mice including in neurons [27, 28]. Likewise, in a murine model of congenital toxoplasmosis, infection resulted in a decrease in neuron number and markers of apoptosis were found indicating infection induced apoptosis in CNS tissues [29]. In murine neural stem cells, infection was also found to induce apoptosis although only apoptosis of neighboring neurons were assessed in this study [30]. A study in murine brain indicated infected neurons were resistant to apoptosis and apoptosis was only induced in the uninfected neurons [31]. Microglia cells which are activated in the brains of mice infected with *T. gondii* with toxoplasmic encephalitis produced nitric oxide (NO), which induces neuronal apoptosis indicating activated microglia may be part of the mechanism leading to neuronal loss of uninfected neurons [32]. Collectively, the above results are suggestive of a mechanism in which *Toxoplasma* infection of neural tissues induces apoptosis of neurons, thus leading to loss of neurons which could lead to neurological abnormalities. However, it is unclear if apoptosis of infected neurons was induced or, alternatively, if infected neurons are resistant to apoptosis. Suppression of apoptosis of infected neuronal cells may facilitate development

of the cysts in neurons and allow persistence of the parasite in the brain. A better understanding of parasite modulation of apoptosis in infected neurons is thus of importance. A better understanding of the modulation of apoptosis in infected neurons could lead to the development of antiparasitic drugs and other therapeutic interventions to control and manage the chronic phase of infection.

#### *3.1.1. Parasite mechanisms modulating apoptosis*

The parasite replicates within a membrane-bound compartment called the parasitophorous vacuole (PV), and hence it is not in direct contact with the host cell cytoplasm and regulators or effectors of apoptosis. However, parasite secretory molecules, which are either released into the host cell at invasion, secreted across the PV membrane (PVM) or act at the PVM cytosolic surface, have been identified which can modulate host cell apoptosis [25, 33]. These mechanisms are briefly summarized below.

#### *3.1.2. Parasite mechanisms that stimulate apoptosis*

Several mechanisms have been identified by which the parasite can stimulate apoptosis. In parasite-infected murine macrophages, supernatants can induce apoptosis of neighboring uninfected macrophages via nitric oxide [34]. Induction of apoptosis has also been found to occur via nitric oxide secretion from activated microglia. Nitric oxide secretion from activated microglia can lead to neuronal cell death and may be an important mechanism by which infection leads to neuronal loss in the brain. The parasite secretory molecule, GRA1, has been identified as capable of inducing apoptosis of infected monocytes and uninfected bystander cells [35]. Secreted GRA1 from sites of ongoing *T. gondii* replication could also induce apoptosis of monocytes recruited to the site of parasite replication. Given monocytes are essential to control parasite replication at the site of primary infection, inhibition of apoptosis in monocytes could serve to downregulate host responses early in infection, helping to establish the infection in the host.

#### *3.1.3. Parasite mechanisms that inhibit apoptosis*

Multiple mechanisms have been identified by which the parasite can inhibit apoptosis [25]. Activation of NF-κB is a mechanism that inhibits apoptosis in fibroblasts and some other host cells [36–38]. Early after infection, the parasite induces rapid translocation of host cell transcription factor, NF-kB, into the nucleus, which activates cell survival pathways and induction of an anti-apoptotic state of the host cell. Nuclear translocation of NF-κB and subsequent gene expression requires activity of the host IκB kinase (IKK). The activation of NF-κB was associated with localization of phosphorylated IκB$\alpha$ subunits to the PVM that was mediated by a parasite-derived IκB kinase (TgIKK). TgIKK is produced as the parasite replicates, and thus allows for a continued phosphorylation of IκB and sustained inhibition of apoptosis. Thus, *T. gondii* modulation of NF-κB gene expression and induction of anti-apoptotic state of the host cell involves both the host cell IKK and TgIKK activity at different phases of infection. NF-kB

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activation has not been found to occur in infected macrophages. Rather in macrophages, *T. gondii*, inhibits apoptosis by $G_i$-protein-mediated signaling, activating PI 3-kinase leading to phosphorylation of protein kinase B (PKB/AKt) as an inhibitor of apoptosis [39]. A micro-RNA-mediated mechanism has also recently been identified in macrophages that inhibited apoptosis via a reduction in Bim, a pro-apoptotic effector of the Bcl-2 family [40]. Other mechanisms have been identified in various cell types that can inhibit caspase cascade including blockage of caspases 8 and 9 and 3, increased expression of anti-apoptotic molecules of the Bcl-2 family, decreased activity of poly (ADP)-ribose polymerase and inhibition of granzymes [33, 41].

Only a few parasite proteins that modulate apoptosis have been identified. The parasite serine–threonine phosphatase, called TgPP2C, was recently identified which downregulates apoptosis in host cells [42]. TgPP2C is secreted into host cells from the PV and translocates to the host cell nucleus and has been shown to regulate growth and survival of the parasite. Using a yeast two-hybrid system, TgPP2C was found to interact with host cell protein, SSRP1 (structure-specific recognition protein 1), which binds to DNA and regulates DNA repair genes. It is speculated that SSRP1 might also be involved in expression of other genes involved in cell survival and apoptosis. Identification of other parasite molecules by which the parasite manipulates host cell apoptosis would facilitate a deeper understanding of host–parasite relationship and may lead to development of new therapeutic targets and antiparasitic drugs.

#### *3.1.4. Parasite modulation of apoptosis: significance to pathogenesis*

In conclusion, *T. gondii* has the ability to both promote and inhibit apoptosis of its own host cell and of uninfected neighboring cells by multiple mechanisms. The ability to modulate apoptosis varies by host cell type, parasite load and parasite virulence. Manipulation of apoptosis has been proposed to be crucial to promoting a stable parasite–host interaction and allowing establishment of persistent infection [23]. The multitude of anti-apoptosis mechanism that the parasite *T. gondii* employs is likely reflective of its status as an obligate intracellular parasite and the essentiality of maintenance of host cell viability to sustain parasite replication and survival. However, intracellular replication eventually leads to lysis of the host cell and typically occurs between 48 and 72 h post-infection, a temporal scale similar to apoptosis. Given the similar temporal scales of lytic death of the host cell and cell death by apoptosis, it can be argued inhibition of apoptosis of the host cell is not necessary to allow for parasite replication. However, as the parasite needs to obtain purine, cholesterol, tryptophan and other components from the host cell, the parasite inhibition of host cell apoptosis may also serve to maintain the host cell in a pro-survival state to enhance availability of host cell nutrients. Inhibition of apoptosis may also be critical for the chronic phase of infection allowing differentiation of bradyzoites and development of the cyst stage in neurons. A better understanding of manipulation of apoptosis by bradyzoites in host cells and, specifically in neurons, is of great importance as the CNS is the compartment in which the parasite predominantly persists in the chronic infection and the cause of serious clinical sequelae in the CNS of immunocompromised individuals causing reactivated toxoplasmosis, in immature immune system of the fetus and newborns causing congenital toxoplasmosis, and in immunocompetant individuals possibly contributing to the development of serious neuropsychiatric disorders.

### 3.2. Autophagy

Autophagy has been found to play a role in host defense against many intracellular pathogens as the autophagy pathway can degrade intracellular pathogens via autophagolysosomes including *T. gondii* [16]. *T. gondii* replicates within a membrane compartment in the host cell called the parasitophorous vacuole (PV) that is a non-fusogenic compartment and does not fuse with cell lysosomes. However, the PV can be delivered to the lysosomes via autophagy-mediated delivery in IFN-$\gamma$-activated macrophages resulting in killing of the parasite [43]. This autophagic-mediated parasite killing is mediated by the immunity-related GTPases (IRGs) which are stimulated by IFN-$\gamma$. Engagement of CD40 also has been found to induce killing of *T. gondii* via autophagy in many nonhematopoetic cells including endothelial cells lines, human and mouse retinal pigment epithelial cells as well as in hemopoietic cells such as macrophages [44, 45].

Interestingly, in non-immune-stimulated cells, evidence indicates the parasite can use autophagy to enhance its own survival. In the first 24 h after infection, host cell autophagy is upregulated and the parasite uses autophagy to acquire host cell nutrients, while at 24–36 h after infection when significant parasite replication has occurred, host cell autophagy is suppressed [46, 47]. This biphasic response may be due to the crosstalk that exists between apoptosis and autophagy. That is, inhibition of apoptosis occurs in infected cells during the first 24 h after infection and as a result of crosstalk, it has been suggested that autophagy is stimulated and then, vice versa, during the later stages of the intracellular infection cycle (> 24 h post-infection), as apoptosis inhibition wanes, suppression of autophagy increases. Thus, regulation of host cell autophagy by the parasite may be part of a cell survival mechanism in *T. gondii* infected host cells related to inhibition of apoptosis. Interestingly, parasite-mediated activation of AKt signaling has recently been found to prevent autophagy degradation of the parasite [48]. As AKt activation has also been linked to inhibition of apoptosis in *T. gondii* infected cells, this event suggests AKt signaling may be a pathway by which the parasite can regulate both host cell autophagy and apoptosis pathways [39, 48]. Dual manipulation of host cell apoptosis and autophagy pathways indicates a fine level of control of host PCD pathways by the parasite to promote parasite persistence in the host. Further work on the interplay between apoptosis and autophagy and the parasite modulation of these two processes is needed to further elucidate these mechanisms.

### 3.3. Necrotic cell death

A few *in vitro* studies have reported *T. gondii* inducing necrotic cell death of host cells [49–52]. In IFN-$\gamma$ stimulated fibroblasts and astrocytes, infection with avirulent parasite strains of the parasite, rather than stimulating autophagic-mediated mechanism of parasite killing, induces disruption of the PV membrane via IRG proteins resulting in parasite death and subsequently triggering of necrotic host cell death [50, 53]. This type of necrotic death of the host cells involved killing of intracellular parasites and thus would promote host survival via elimination of the parasite. In these experiments, the parasite was found to egress within hours after infection, thus limiting replication in host cells which also would promote clearance of the parasite by immune cells. Experiments have similarly found early egress of the parasites in

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IFN-$\gamma$-stimulated astrocytes, indicating this mechanism may also function in the brain to limit parasite numbers and aid in elimination of the parasite [54].

Conversely, *Toxoplasma*-specific primed T cells were found to be able to induce rapid egress of the parasite, causing necrotic cell death of host cells via ligation of the death receptor or perforin, but as egress was rapid the intracellular parasites escaped killing [55]. These egressed parasites were capable of infecting neighboring cells, thus suggesting that during *Toxoplasma* infection of T cells, death receptor-and perforin-mediated parasite egress may contribute to parasite dissemination of the parasite. Furthermore, as necrotic cell death induced by death receptor or perforin-dependent parasite egress contributes to inflammatory processes, it could also lead to further spread of the parasite due to infection of invading leukocytes. This process may also contribute to the establishment of infection in the brain as infected leukocytes can invade brain. Finally, death receptor-induced or perforin-mediated egress could be involved during the reactivation of *T. gondii* in chronically infected individuals, leading to reinfection of egressed parasites in the brain. Thus, parasite evasion of this cell death pathway may have significant consequences on the clinical sequelae of the chronic infection in the brain. Experiments in fibroblasts found necrotic host cell death was not dependent on the necroptosis mediator RIPK3 or caspases [52]. Further studies are needed to ascertain if necroptosis occurs in other cell types that have been associated with necrotic-type cell death in *T. gondii* infected cells.

## 4. Parasite manipulation of PCD pathways: Conclusions and potential therapeutic implications

A better understanding of the mechanisms by which the parasite modulates apoptosis, autophagic and necrotic host cell death pathways will enhance our understanding of the host/parasite relationship in toxoplasmosis and may yield new therapeutic targets to treat pathological consequences of cerebral toxoplasmosis. For example, while the infection is asymptomatic in immunocompetant individuals, recent evidence indicates chronic infection may lead to psychiatric and other neurological disorders in some individuals. Some of these effects may be due to modifications of processes leading to cell death or resulting in dysregulated cell death, resulting in reduction in CNS cell numbers and specifically of neurons. Conversely, inhibition of apoptosis of astrocytes in the brain could be crucial for establishment of the parasite in this compartment, while inhibition of apoptosis of infected neurons may be essential for development of bradyzoite stage and thus crucial to long-term persistence of the parasite in the brain. The evidence indicates the parasite can manipulate both host cell apoptosis and autophagy pathways, indicating a sophisticated level of parasite control of host PCD pathways and elucidation of the molecular details of these mechanisms could lead to new therapeutic targets with which to control the infection. A better understanding of manipulation of apoptosis and other cell death pathways in the brain cells such as microglia, astrocytes and neurons is of particular importance. The identification of molecular components of PCD pathways crucial to parasite–host interactions in chronic toxoplasmosis could lead to development of new antiparasitic drugs and/or yield new potential therapeutic targets to treat

pathological consequences of chronic cerebral toxoplasmosis in immunocompetant individuals and reactivated toxoplasmosis in immunosuppressed individuals. A similar harnessing of apoptotic, autophagic and programmed necrosis cell death pathways has been proposed to treat cancers, neurodegenerative disorders and other diseases, where PCD pathways have been identified as involved in pathogenesis [56]. The dissection of the molecular mechanisms by which *T. gondii* manipulates apoptosis and other cell death pathways thus could also serve as a benefit to probe pathways in normal cells and in diseases in which apoptosis and other cell death pathways play a central role.

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Section 3

# Necrosis

Chapter 18

# HMGB1 in Cell Death

Daolin Tang and Rui Kang

Additional information is available at the end of the chapter

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**Abstract**

High mobility group box 1 (HMGB1) is named for its electrophoretic mobility on polyacrylamide gels when it was first identified in calf thymus in 1973. HMGB1 plays a critical role in the stress response not only inside the cell as a DNA chaperone and cell death regulator, but also outside the cell as the prototypic damage-associated molecular pattern molecule. The physiological and pathological role of HMGB1 in health and disease has been widely studied for years. In this chapter, we will focus on the release and function of HMGB1 in cell death types such as apoptosis, autophagy, and necrosis.

**Keywords:** hmgb1, autophagy, necrosis, apoptosis

## 1. Introduction

Cell death is the cell's process of losing its biological ability to carry out all the essential life processes. The Nomenclature Committee on Cell Death proposes several cell death classification criteria. According to morphological appearance, cell death is divided into apoptosis (type I), autophagy (type II), and necrosis (type III) [1, 2]. According to enzymological qualities, cell death is divided into several subtypes depending on the involvement or noninvolvement of nuclei or distinct protease classes such as caspases, calpains, cathepsins, and transglutaminases. According to immunological characteristics, cell death is divided into immunogenic or tolerogenic cell death [3]. For example, apoptosis is generally considered nonimmunogenic cell death, whereas necrosis is considered immunogenic cell death. In addition, cell death can be classified into regulated or accidental cell death based on functional aspects [4]. Accidental cell death is caused by unexpected and accidental cell damage (e.g., ischemic and trauma), whereas regulated cell death is mediated by an expected program in response to different stimuli. The list of regulated cell death subtypes is rapidly increasing and includes anoikis, autophagic cell death, apoptosis, cornification, entosis, ferroptosis, mitotic catastrophe,

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necroptosis, netosis, parthanatos, and pyroptosis [4]. Cell death is essential for a plethora of physiological processes, and its deregulation is implicated in several human diseases such as infections, neurodegeneration, cancer, autoimmunity, and ischemic disease [5-7]. During the past few decades, a number of important concepts regarding the regulation of cell death and its roles in human health and disease have arisen. Understanding the molecular mechanisms and signaling pathways of cell death is crucial for identifying new diagnostic and therapeutic targets.

Compared to pathogen-associated molecular pattern molecules (PAMPs), which are generated from the components of foreign pathogens such as bacteria and viruses, damage-associated molecular pattern molecules (DAMPs) are endogenous or self-molecules that are secreted, released, or undergo surface exposure by dead, dying, or injured cells [8-12]. Both PAMPs and DAMPs are mainly recognized by pattern recognition receptors such as receptor for advanced glycation end products (RAGE) and toll-like receptors (TLRs) to mediate the inflammatory, immunity, and metabolism response. The release and activity of DAMPs during cell death can determine whether cell death is immunogenic or tolerogenic [13]. Thus, DAMPs are suitable emergent targets for cell-death-associated immune therapy.

High mobility group box 1 (HMGB1) is named for its electrophoretic mobility on polyacrylamide gels when it was first identified in calf thymus in 1973 [14]. As an extremely conserved protein, HMGB1 originated before the divergence of the protostomes and deuterostomes, approximately 525 million years ago [15]. HMGB1 shares 100% amino acid sequence identity between mice and rats, and a 99% homology between rodents and humans [16-18]. The homolog of mammalian HMGB1 has been reported for several species such as *Nhp6A/B* in yeast and *HMG-D* and *DSP1* in *Drosophila* [19-21]. HMGB1 plays a critical role in the stress response not only inside the cell as a DNA chaperone and cell death regulator, but also outside the cell as the prototypic DAMP. The physiological and pathological role of HMGB1 in health and disease has been widely studied for years [22]. In this chapter, we will focus on the release and function of HMGB1 in cell death types such as apoptosis, autophagy, and necrosis.

## 2. HMGB1 structure and function

### 2.1. Structure

Human HMGB1 consists of 215 amino acid residues and has two L-shaped DNA-binding domains (HMG A box [9-79aa], HMG B box [95-163aa]) and a shorter C-terminal tail (186-215aa) [23]. Both A- and B-box domains are necessary for efficient DNA bending and flexure. HMGB1 binds to DNA without apparent sequence specificity. HMGB1 normally locates in the nucleus due to two nuclear-localization signals (NLS): NLS1 (28-44aa) and NLS2 (179-185aa) [24]. In contrast, HMGB1 contains nuclear-emigration signals in DNA-binding domains, which contributes to extranuclear HMGB1 during stress in a nuclear exportin chromosome-region maintenance 1-dependent manner. In addition to DNA, HMGB1 can bind a number of proteins involved in multiple biologic processes. For example, HMGB1 binds to RAGE, TLR4, and p53 by residues 150-183, 89-108, and 7-74, which mediates cell migration

[25], cytokine production [26], and gene transcription [27], respectively. The recombinant B box protein exhibits proinflammatory activity, whereas the recombinant A box protein displays anti-inflammatory activity [28], although the potential mechanism remains unknown. The C terminus is composed of 30 acidic amino acid residues and is able to regulate DNA binding/bending by intramolecular interaction with the A- and B- box [29, 30] or by intermolecular interaction with histones (e.g., H1 and H3) [31, 32]. Additionally, residues 201-205 in the C-terminal acidic tail region contribute to the antibacterial activity of recombinant HMGB1 [33]. Hence, the structural basis of HMGB1 determines its biological function.

### 2.2. Intracellular HMGB1

#### *2.2.1. Nuclear HMGB1*

HMGB1 translocates between the cytoplasm and the nucleus, but normally stays in the nucleus in most cells and tissues. Nuclear HMGB1 is the structural protein of chromatin and orchestrates a number of nuclear events by its DNA chaperone activity as follows: (1) Nucleosome stability and sliding. As basic unit of chromatin, nucleosome contains a short length of DNA wrapped around a core of histone proteins. HMGB1 and histone H1 can bind to linker DNA between successive nucleosomes in the chromatin fiber [34]. H1 stabilizes nucleosome with less mobility, whereas HMGB1 relaxes nucleosome and makes chromatin more accessible at the distorted site [35, 36]. (2) Nucleosome number and genome chromatinization. Loss of HMGB1 in mammalian and yeast cells leads to 20-30% less histones and nucleosomes and more RNA transcripts [37]. (3) Nuclear catastrophe and nucleosome release. Conditional knockout of HMGB1 in the pancreas causes nuclear oxidative injury and proinflammatory nucleosome release, which mediates sterile inflammation [38]. (4) DNA bending and binding. HMGB1 binds to DNA with structure-specificity, but not sequence-specificity [39]. After binding DNA, the major function of HMGB1 is to bend and change DNA conformation by unwinding [40], looping [41], or compacting DNA [42]. This DNA chaperone activity of HMGB1 is implicated in the regulation of gene transcription [43], DNA repair [44], DNA replication [45], V(D)J recombination [46], gene delivery [47], and gene transfer [48]. (5) Telomere homeostasis. Loss of HMGB1 in yeast and mammalian cells inhibits telomerase activity, decreases telomere length, and increases DNA damage and chromosomal instability [49].

#### *2.2.2. Cytosolic HMGB1*

Several cell types (e.g., fibroblasts [50], thymocytes [51]), and tissue types (e.g., liver, kidney, heart, and lung) [52] have normal cytosolic HMGB1 expression. The ratio of nuclear to cytoplasmic HMGB1 is about 30:1 [52]. Importantly, the translation of HMGB1 from the nucleus to the cytosol, including mitochondria and lysosomes, are observed in response to various stressors (e.g., cytokines, chemokines, heat, hypoxia, oxidative stress, and oncogenes). Although the function of cytosolic HMGB1 still remains poorly studied, HMGB1 may act as a positive regulator of mitochondrial quality in an autophagy-dependent and autophagy-independent manner [53, 54], which will be discussed later in the "Autophagy" section. In addition to autophagy, cytosolic HMGB1 is involved in the regulation of unconventional

secretory pathways based on mass spectrometry-mediated binding partner analysis [55]. In one study, several HMGB1-binding partners in nuclear and cytosol fraction were identified in several cancer cells [55]. Among them, nine of the cytosolic HMGB1-binding proteins were related to protein translocation and secretion. In particular, immunoprecipitation analysis further confirmed four cytosolic HMGB1-binding proteins, including annexin A2, myosin IC isoform a, myosin-9, and Ras-related protein Rab10 [55]. These proteins are directly implicated in the process of unconventional protein secretion. Further studies are needed to define the function of cytosolic HMGB1 in unconventional protein secretion. In addition to nuclear and cytosolic HMGB1, intracellular HMGB1 presents on cell surface membranes and regulates neurite outgrowth [56], platelet activation [57, 58], cell differentiation [59], erythroid maturation [60], adhesion [61], and innate immunity [62].

### 2.3. Extracellular HMGB1

HMGB1 is released in two different ways. On the one hand, HMGB1 can be actively secreted by normal cells, especially immune and endothelial cells [63, 64]. On the other hand, HMGB1 can be passively released by dead, dying, or injured cells in response to autophagic cell death [65], apoptosis [66, 67], necrosis [68], necroptosis [69, 70], netosis [71], and pyroptosis [72]. Oxidative stress refers to elevated intracellular levels of reactive oxygen species (ROS) that play a central role in the regulation of HMGB1 secretion and release, although the actual mechanism of action remains ambiguous [73]. Once released, HMGB1 acts as a cytokine, chemokine, and growth factor that is implicated in multiple biological processes including inflammation, immunity, migration, invasion, metabolism, proliferation, differentiation, antimicrobial defense, angiogenesis, tissue regeneration, death, autophagy, senescence, and efferocytosis. Extracellular HMGB1 plays important roles in the pathogenesis of human disease and is a potential therapy target in infection and sterile inflammation [74-76]. Several factors can affect HMGB1 activity in different experimental settings. For example, RAGE [77] and TLRs [78, 79] are positive receptors in macrophages and fibroblasts, whereas CD24 [80] and T cell immunoglobulin mucin 3 [81, 82] are negative receptors of HMGB1-mediated signaling in macrophages and dendritic cells (DCs). In addition to receptors, HMGB1 can be directly taken up and mediate the inflammatory and metabolism response [83, 84]. Ultra-pure HMGB1 (free from contaminating bacterial proteins and nucleic acids) exhibits very low immune activity in macrophages. In contrast, extracellular HMGB1 is in fact a “sticky” protein and a synergistic immune effect is observed between HMGB1 and PAMPs (e.g., lipopolysaccharide), DAMPs (e.g., DNA), and other molecules (e.g., cytokines, chemokine, and IgG) in multiple cells [85]. Thus, serum and plasma components (e.g., immunoglobulins, phospholipids, thrombomodulin, and proteoglycans) can interfere with HMGB1 detection by enzyme-linked immunosorbent assay [86]. Another important factor affecting HMGB1 activity is its redox status [87]. HMGB1 contains three conserved redox-sensitive cysteine residues: C23, C45, and C106. Reduced all-thiol-HMGB1 only exhibits chemokine activity, whereas disulfide-HMGB1 displays only cytokine activity, and oxidized HMGB1 has neither in immune cells [88]. In addition, reduced HMGB1 induces autophagy, whereas oxidized HMGB1 triggers apoptosis in cancer cells [89]. This redox status of HMGB1 also affects the affinity between HMGB1 and its receptors [26]. A recent study demonstrates that HMGB1 is specifically cleaved

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by caspase-1 but not other caspases during inflammasome activation [90]. Collectively, the release and activity of HMGB1 is context-dependent.

# 3. HMGB1 regulates cell death

## 3.1. Mechanism of HMGB1-mediated autophagy regulation

Autophagy, including macroautophagy, microautophagy, and chaperone-mediated autophagy, is a highly conserved degradation process in organisms from yeasts to plants and animals [91]. The well-studied form of autophagy is macroautophagy (hereafter referred to as autophagy). As a complex dynamic process, autophagy is composed of the formation and maturation of three major membrane structures: the phagophore, autophagosome, and autolysosome [92]. Briefly, the phagophore originates from multiple membrane resources and engulfs the cytosolic materials, which leads to the formation of a closed autophagosome with a double membrane. Of note, microtubule-associated protein light chain 3 (LC3)-II is a widely used autophagosome marker [93]. Finally, autophagosomes fuse with lysosomes to form autolysosomes, which results in degradation of the engulfed material, including LC3-II, by lysosomal enzymes into elementary pieces that can be used for protein synthesis and energy production. Thus, autophagy is a programmed cell survival pathway in response to intracellular and extracellular stress [94]. However, excessive or impaired autophagy can cause cell death, indicating a dual role of autophagy in cell survival and cell death. In particular, autosis is an $Na^+$, $K^+$-ATPase-dependent form of cell death triggered by autophagy-inducing peptides, starvation, and hypoxia–ischemia [95]. The process of autophagy is controlled by multiple posttranslational modifications of the autophagy-related gene (Atg) family and shares regulators derived from other cell death pathways [96].

HMGB1 promotes autophagy in a location- and modification-dependent manner. Nuclear HMGB1 regulates heat shock protein β-1 (HSPB1) expression at a transactional level [54]. The protein expression of HSPB1, but not other heat shock proteins, is significantly inhibited in HMGB1 knockout or knockdown cells. Both HMGB1 and HSPB1 regulate mitochondrial selective autophagy, namely mitophagy, following mitochondrial injury [54]. Like other ATGs, it was recently suggested that HMGB1-independent autophagy exists in the regulation of mitochondrial quality, including the mitochondrial DNA damage response [53]. Cytosolic HMGB1 is a Beclin-1 binding protein [97]. HMGB1 C23S and C45S mutants lose their ability to bind Beclin-1 and therefore cannot promote autophagy [97]. The binding of HMGB1 with Beclin-1 is positively regulated by unc-51-like kinase 1 [98] mitogen-activated kinase-like protein [99], and nucleus accumbens-1 [100]. In contrast, p53 [101], SNCA/$\alpha$-synuclein [102], lysosomal thiol reductase [103], miR34A [104], and miR22 [105] negatively regulate HMGB1-mediated autophagy by disrupting HMGB1-Beclin-1 complex formation. Moreover, activation of poly [ADP-ribose] polymerase 1 (PARP1) is required for tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced ADP-ribosylation of HMGB1 and subsequent HMGB1-Beclin-1 complex formation in cancer cells [106]. Extracellular reduced HMGB1, but not oxidized HMGB1, significantly induced autophagy in cancer cells in a RAGE-dependent manner [89]. This process may sustain anaerobic energy

production during tumor growth and development [107]. Collectively, these findings suggest an HMGB1-dependent autophagic pathway at multiple levels in response to stress. However, HMGB1-independent autophagy may exist in several organs, although the underlying mechanism of its action remains obscure [108].

### 3.2. Mechanism of HMGB1-mediated apoptosis regulation

Apoptosis is the process of programmed cell death and includes classical “extrinsic” and “intrinsic” pathways and nonclassical T and natural killer cell-mediated cytolytic pathways. The extrinsic pathway is primarily mediated by the binding of a ligand to a transmembrane death receptor (DR). DRs are members of the TNF receptor gene superfamily, including FasR, TNFR1, lymphotoxin receptor, DR3, and DR4/DR5 [109]. In addition to DRs, dependence receptors mediate apoptosis by monitoring the absence of certain trophic factors or the presence of anti-trophic factors [110]. The intrinsic pathway for apoptosis involves activation of a mitochondrial pathway including altering mitochondrial permeability and subsequent release of mitochondrial proteins such as cytochrome c and second mitochondrial-derived activator of caspases [111]. The process of apoptosis is tightly regulated by the Bcl-2, caspase, and nuclease families [112-114]. Caspases are a family of endoproteases linking inflammation and cell death. Initiator caspases (e.g., caspases-8 and -9) activate executioner caspases (e.g., caspases-3, -6, and -7) that mediate the cleavage of key structural proteins such as PARP1. However, caspase-independent apoptosis may exist by translocation of apoptosis-inducing factor [115, 116] and endonuclease G [117] from the mitochondria to the nucleus, or activation of Omi/HTRA2 (a mitochondrial serine protease) [118]. Remarkably, several caspases (e.g., caspase-1, -4, -5, and -12 in humans; caspase-1, -11, and -12 in mice) are critical mediators of innate immune responses partly by activation of inflammasome, but not activation of the apoptosis pathway.

Intracellular HMGB1 is generally an anti-apoptotic protein in response to several apoptotic stimuli such as ultraviolet radiation, CD95, TRAIL, caspase-8, and Bax [119]. Knockdown of HMGB1 increases drug sensitivity in cancer cells [120]. Mechanically, HMGB1 plays transcriptional-dependent (e.g., regulation of Bcl-2 family protein expression) and transcriptional-independent roles (e.g., regulation of autophagy and p53 location) in the regulation of apoptosis. For example, inhibition of HMGB1-mediated autophagy can increase caspase activity [121]. In addition to caspases, several non-caspase proteases such as calpain ($Ca^{2+}$-dependent proteases) may play a role in the execution of apoptosis. Interestingly, HMGB1 deletion can enhance calpain activity and trigger cleavage of Beclin-1 and ATG5 [122]. Thus, HMGB1 is an important regulator of the cross talk between apoptosis and autophagy. *In vivo*, conditional knockout of HMGB1 in pancreas, liver, intestinal epithelial and myeloid cells enhances sterile inflammation and infection partly through inhibition of autophagy and induction of apoptosis [38, 122-124]. In some cases, overexpression of HMGB1 renders cells sensitive to apoptosis in response to chemotherapy agents [125]. In addition, extracellular oxidized HMGB1 can induce caspase-dependent apoptosis in cancer cells [89]. These findings suggest that HMGB1 plays dual roles in the regulation of apoptosis.

### 3.3. Mechanism of HMGB1-mediated necrosis regulation

Necrosis includes accidental and regulated necrosis [2]. Partially, the term "necroptosis" has recently been used to describe regulated necrosis when cells lack the capacity to activate caspase [126]. Necroptosis is mediated by a signaling complex called necrosome, containing receptor-interacting protein (RIP)1, RIP3, and mixed-lineage kinase domain-like (MLKL) [127, 128], and can be inhibited by small molecule inhibitors necrostatin 1 and necrosulfonamide [129, 130] [126]. The fundamental causes of necrosis include calcium overload, ROS generation, cellular energy depletion, and membrane lipid injury [131]. PARP is a protein family involved in a number of cellular processes such as DNA repair and programmed cell death. Induced overactivation of PARP1 can lead to adenosine triphosphate (ATP) depletion and subsequent necrosis [132]. The process of necrosis ends with the leaking out of enzymes from lysosomes to digest cell components that are associated with HMGB1 release. *In vivo*, loss of HMGB1 in the pancreas increases L-arginine-induced apoptosis and necrosis due to oxidative injury [38]. However, the role of HMGB1 in necroptosis remains undefined.

## 4. HMGB1 release in cell death

### 4.1. Mechanism of HMGB1 release in autophagy

Autophagic cell death is not only a morphologic notion such as cell death associated with autophagosomes and autolysosomes, but also a functional description that excessive autophagy can cause cell death. Induction of autophagy facilitates both active secretion and passive release of HMGB1. For example, the release of HMGB1 is significantly increased in response to epidermal growth factor (EGF) receptor-targeted diphtheria toxin (DT-EGF)-induced autophagic cell death [65]. In contrast, suppression of ATG5, ATG7, or ATG12 expression by RNA interference (RNAi) inhibits autophagy and subsequent HMGB1 release after treatment with DT-EGF in cancer cells [65]. In addition, ATG5-dependent autophagy promotes HMGB1 secretion in fibroblasts and macrophages after treatment with Hank's balanced salt solution and lipopolysaccharide [97, 133]. Antioxidant (e.g., N-acetyl-L-cysteine) inhibits the cytosolic translocation and release of HMGB1 in starvation-induced autophagy [97]. In contrast, ROS and knockdown of superoxide dismutases (SOD)-1 and SOD2 by RNAi promotes cytosolic HMGB1 expression and extracellular release [134]. These findings suggest that oxidative stress is involved in autophagy-mediated HMGB1 release.

### 4.2. Mechanism of HMGB1 release in apoptosis

An early study indicated that HMGB1 is released only by necrotic cells, but not apoptotic cells [68]. However, recent studies demonstrated that activation of caspases and deoxyribonuclease (DNase) in apoptosis regulates HMGB1 release and activity in apoptosis. Caspase-3 and caspase-7 are important executioner caspases in apoptosis through amplified initiation signals from caspase-8 and caspase-9. Activation of caspase-3 and -7 induces mitochondrial complex 1 protein p75 NDUFS1 cleavage, which results in mitochondrial ROS production and subse-

quent HMGB1 release during apoptosis in DCs [135]. Interestingly, the activity of released HMGB1 in apoptosis is impaired, which promotes immunological resistance due to its oxidized form [135]. In addition to caspase-3 and -7, caspase-1 is responsible for HMGB1 cleavage and release in the response to pyroptosis in immune cells [72, 136, 137]. This caspase-1-mediated HMGB1 fragment can rescue apoptosis-induced immune tolerance in a RAGE-dependent manner [137]. Thus, different caspases can determine HMGB1 release and action in apoptosis and pyroptosis.

DNase is responsible for DNA fragmentation during cell death. Activation of DNA endonuclease (DNase-gamma) contributes to the degradation of DNA into nucleosomal units in apoptosis, whereas activation of DNase I and II facilitates degradation of DNA in necrosis [138]. The release of HMGB1 in apoptosis is triggered by DNase-gamma-mediated nucleosomal DNA fragmentation [139, 140]. Thus, inhibition of DNase gamma activity by small molecular compound DR396 can significantly diminish HMGB1 release in response to apoptotic stimuli [139, 140].

### 4.3. Mechanism of HMGB1 release in necrosis

The nuclear enzyme PARP1, which catalyzes the synthesis of the biopolymer poly(ADP-ribose), exhibits an essential role in the DNA damage response and genomic stability. However, overactivation of PARP1 may deplete the stores of cellular NAD+, which results in ATP depletion and subsequent necrosis [141]. In fact, HMGB1 release in necrosis is regulated by PARP1. Genetic and pharmacologic inhibition of PARP1 inhibits alkylating DNA damage agent-mediated necrosis as well as HMGB1 release [142]. In addition to necrosis, activation of PARP1 also contributes to HMGB1 translocation and release in autophagy and inflammation [106, 143]. Interestingly, loss of HMGB1 in tissue and cells accelerates DNA damage that results in PARP1 overactivation [144]. These findings suggest interplay between HMGB1 and PARP1 in response to cell death.

The RIP3-mediated signaling pathway is responsible for HMGB1 release in necroptosis. Upregulation of RIP3 expression *in vitro* triggers necroptosis, whereas suppression of RIP3 expression by RNAi *in vitro* or *in vivo* significantly inhibits inflammatory stimuli-induced necroptosis. RIP3-deficient mice exhibit resistance to sepsis and donor kidney inflammatory injury. This anti-inflammatory function of RIP3 is due partly to inhibition of HMGB1 and release of other DAMPs [145]. Additionally, RIP3-mediated necroptosis also contributes to dsRNA/poly (I:C)-induced HMGB1 release [146]. This process promotes retinal degeneration and triggers an inflammatory response in the mouse retina [146]. In addition to RIP3, interferon-β promoter stimulator 1 (an adaptor molecule for RIG-I-like receptors) may be critical for poly (I:C)-induced HMGB1 release in necroptosis in DCs.

Cysteine cathepsins are lysosomal proteases with housekeeping functions that also initiate a specific cell death pathway termed lysosomal cell death. This type of cell death includes morphological features of necrosis and apoptosis [147]. Cathepsin B, a critical lysosomal cysteine protease, mediates HMGB1 release following *L. pneumophila*-induced lysosomal cell death [148]. Mechanically, cathepsin B can translocate from the lysosome to the nucleus, where it interacts with HMGB1 and inhibits its cytosolic translocation. In addition to lysosomal cell

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death, cathepsin B is also important for HMGB1 release during inflammasome activation [149, 150]. In contrast, cathepsin D may facilitate HMGB1 release in necroptosis in DCs. The function of other cathepsins in the regulation of HMGB1 release remains unknown.

## 5. Concluding remarks

HMGB1 is a member of family containing the evolutionarily conserved HMG box domains. The function of HMGB1 depends on its cellular location. Besides its functions in the nucleus and cytosol, HMGB1 plays a critical role in extracellular signaling associated with multiple biological processes. Both intracellular and extracellular HMGB1 are involved in the regulation of types of cell death such as apoptosis, necrosis, and autophagy. Intracellular HMGB1 regulates cell death in both transactional-dependent or transactional-independent manners. In many cases, HMGB1 is a negative regulator of apoptosis and necrosis, but a positive regulator of autophagy. In addition, the release and activity of HMGB1 in cell death is context-dependent, which may cause immunogenic cell death or tolerogenic cell death. Future studies are needed to define the upstream and downstream signaling of HMGB1 in the regulation of cell death; clarify the interplay and cooperative role of HMGB1 and other DAMPs in the cell-death-associated microenvironment; and develop new therapeutic strategies for targeting HMGB1 in cell-death-associated disorders.

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Chapter 19

# Necrosis as Programmed Cell Death

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Additional information is available at the end of the chapter

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**Abstract**

The process of cell death is the mechanism through which organisms eliminate useless cells. Hence, it is a normal process that maintains homeostasis. Cell removal can be effectuated by several pathways that involve complex and regulated molecular events specific to each type of cell death. Diverse studies have evidenced different types of cell death: apoptosis, autophagy, and necrosis. This chapter presents a brief review of the apoptotic and autophagic cell death processes but focuses attention primarily on necrosis because it has previously been considered an accidental and uncontrolled form of cell death. More recent evidence, however, has shown that, under certain circumstances, necrosis is conducted by a controlled program called necroptosis, which is now included as a programmed cell death process.

**Keywords:** Apoptosis, autophagy, cell death, necrosis, necroptosis

## 1. Introduction

The tissular environment includes a series of signals that maintain the rates of cell proliferation and cell death so as to conserve structural integrity and functionality. Alterations in either one of these processes can cause certain pathologies, such as cancer. The cell death process is an ongoing event during the development of tissues and organs, one that is present right from embryonic development in the form of programmed cell death, which occurs under physiological conditions as a process that requires the active participation of highly regulated mechanisms. Traditionally, apoptosis was synonymous with programmed cell death; however, different routes of cell death, such as autophagy and, more recently, necroptosis, are now included as forms of programmed cell death. Morphologically, each one of these cell death processes has features that make it possible to distinguish among them. The different molecular mechanisms involved in the cell death pathways are responsible for the morphological changes that occur in the affected cells. However, each pathway has specific characteristics;

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for instance, cellular shrinkage is a phenomenon that occurs in apoptosis [1], but is not present in other types of cell death, such as autophagy or necrosis. On the other hand, the extensive presence of vesicles evidences autophagy but does not appear to the same extent in the other types of cell elimination [2]. Necrosis, meanwhile, presents generalized swelling of membranous organelles that leads to cell rupture [3].

## 2. Brief description of two types of programmed cell death: apoptosis and autophagy

Apoptosis, or type I programmed cell death, is the most widely studied of the forms of cell death. Its morphological characteristics can be identified under light microscopy, and include cell shrinkage, compacting of the chromatin, blebbing of the cytoplasmic membrane, and, finally, the formation of apoptotic bodies [1] (Figure 1). Biochemically, apoptosis is characterized by the participation of proteases called caspases, orderly internucleosomal DNA fragmentation, phosphatidylserine externalization, changes in mitochondrial membrane permeability, and the participation of members of the Bcl-2 protein family.

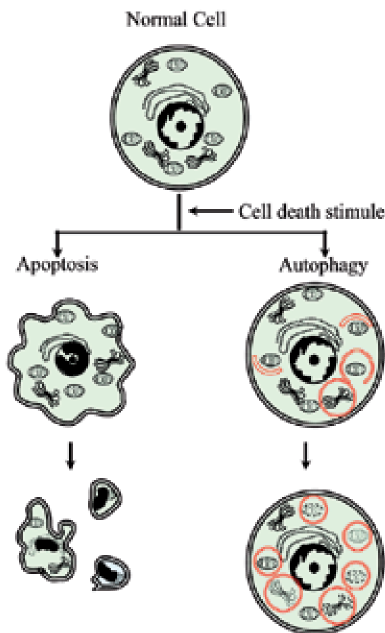


**Figure 1.** Schematic representation of the programmed cell death process type I (apoptosis) and type II (autophagy). Apoptosis is characterized by a cellular contraction, chromatin compaction, membranous blebs, and the formation of apoptotic bodies. Autophagy is characterized by the presence of a large number of autophagosomes with cytoplasmic content. Both types of cell death do not generate an inflammatory response since the cytoplasmic membrane is conserved until the cellular debris are eliminated by neighborhood or by specialized ones.

Caspases are cysteinyl-aspartate-specific proteases that are synthetized in an inactive form as zymogens called pro-caspases (Figure 2). It is this inactive form that allows the controlled execution of the cell death process. Caspases were first identified in the nematode *Ceanorhabditis elegans* [4], but homologous forms are present in mammals [5].

The hallmarks of apoptosis, such as DNA fragmentation and compacted chromatin, result from caspase activity. During apoptosis, DNA is fragmented into nucleosome size (200 bp) [6, 7]. The factor responsible for DNA fragmentation during apoptosis is a specific DNase (CAD, caspase-activated DNase) that is activated by active caspase-3 [8]. Active caspase-3, in turn, is involved in morphological cell changes during apoptosis, where it cleaves rho-associated kinase-1 (ROCK-1) in order to activate it and this, finally, affects the cytoskeletal arrangement causing the apoptotic shrinkage morphology [9].

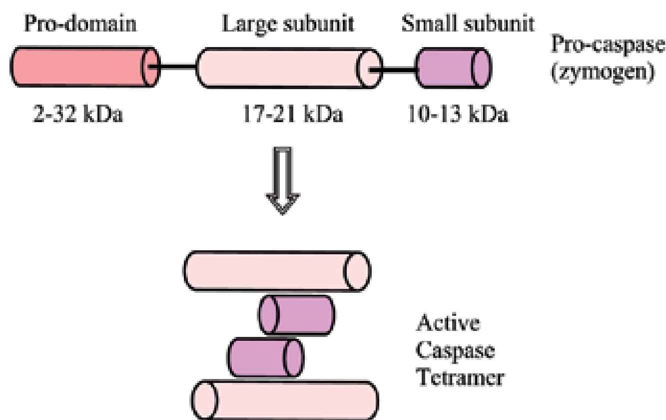


**Figure 2.** Caspases involved in the apoptotic process are synthetized in an inactive form as zymogens, are constituted by a pro-domain, a large subunit, and a small subunit. The zymogens are activated forming tetramers.

Apoptotic cell death is highly regulated by members of the B-cell lymphoma 2 (Bcl-2) family [10]. Bcl-2 family members have been classified as anti-apoptotic and pro-apoptotic proteins according to their Bcl-2 homology (BH) and domain organization (Figure 3). The presence of domains BH1, BH2, BH3, and BH4 corresponds to the group that inhibits apoptosis. The pro-apoptotic group, in contrast, is divided in two groups: those with domains BH1, BH2, and BH3, and those with only the BH3 domains (defined as BH3 only; see the review in [11]). This family of proteins performs its functions at the intracellular level inside the mitochondria, a key element in apoptosis.

Apoptosis can be initiated by two well-described routes: the extrinsic and intrinsic pathways (Figure 4). Extrinsic activation is conducted through the participation of death ligands (such as the tumor necrosis factor – TNF – superfamily, and TNF-related apoptosis-induced ligands, or TRAIL) with their cognate cell surface death receptors (such as TNF receptor 1, Fas, TRAIL receptor 1, or TRAIL receptor 2) (reviewed in [12]). Once the ligand recognizes and bonds to

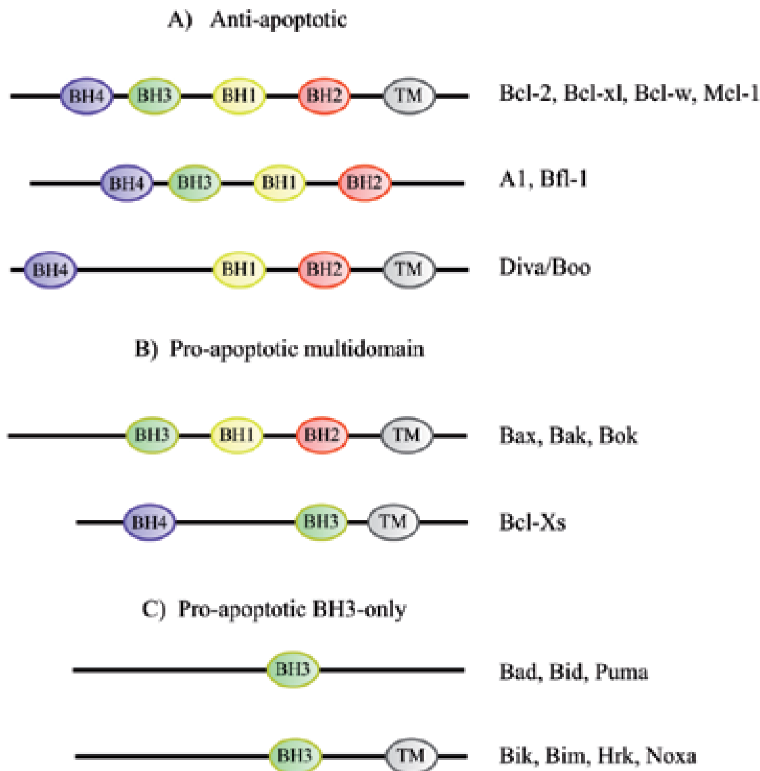


**Figure 3.** B-cell lymphoma 2 (Bcl-2) family proteins. A) General schematization of the structure of Bcl-2 proteins. B) The anti-apoptotic members – they possess all the four BH domains. C) The pro-apoptotic members which in turn are divided into two groups: multidomain and BH3 only.

its receptor, a series of intracellular complexes are formed to activate the initiator caspases (such as -8 and -10), which then activate the executioner caspases (such as -3, -6, and -7). In their activated form, these executioner caspases cleave multiple intracellular targets.

The intrinsic apoptotic pathway, in contrast, can be activated by various stimuli, including DNA damage, growth factor starvation, and oxidative stress [13]. During exposure of cells to these stimuli, the mitochondria are affected, since several members of the Bcl-2 family are activated and promote mitochondria outer membrane permeabilization (MOMP). The permeated external mitochondria membrane allows the release of cytochrome c (cyt c), which is associated with the Apaf-1 protein. The cyt c and Apaf-1 union then bonds to the initiator caspase-9 to form the complex that constitutes the apoptosome, which has the ability to activate the initiator caspases that perform their functions by cleaving specific cellular substrates.

The second process of cell death, autophagy, is a genetically programmed and evolutionarily conserved process that produces the degradation of obsolete organelles and proteins. It is activated by such extracellular stimuli as nutrient starvation, hypoxia, high temperature, and

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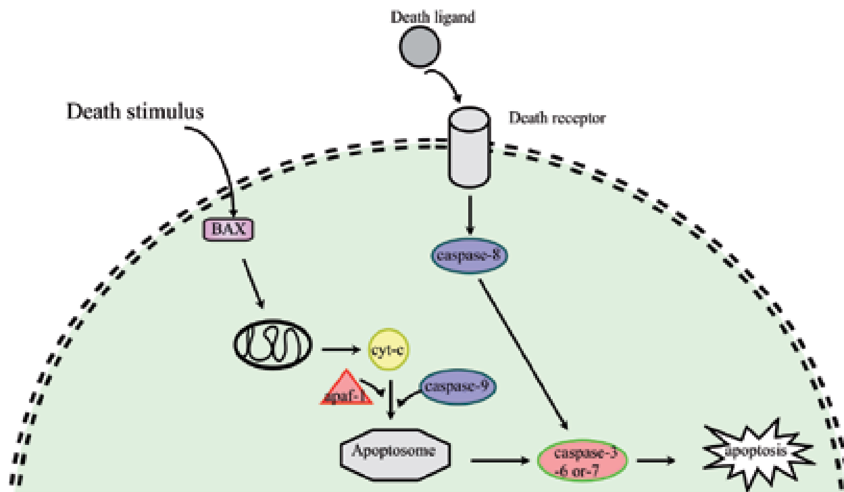


**Figure 4.** Routes of activation of apoptosis. The extrinsic route is mediated by external signals – a ligand – that activate to the membrane receptor. The ligand–receptor interaction induces the assembly of the death-inducing signaling complex (DISC) to promote the activation of caspase-8, which in turn is able to activate to the executor caspase -3, -6, or -7, conducting to the morphological changes of the apoptosis. The intrinsic route is directed by the mitochondrial outer membrane permeabilization, which allows the release of pro-apoptotic elements as cytochrome-C. Cytochrome-C induces the apoptosis protease-activating factor 1 (Apaf-1) to promote the activation of caspase-9 to assemble the apoptosome. The apoptosome is capable of activating to the executor caspases.

altered intracellular conditions, including the accumulation of damaged or superfluous organelles (reviewed in [2]).

In eukaryotic organisms, three types of autophagy have been described: microautophagy, macroautophagy (commonly called simply autophagy), and chaperone-mediated autophagy (Figure 5). Microautophagy involves the engulfing of cytoplasmic components directly at the level of the lysosome by means of an invagination process, while macroautophagy entails the formation of double-membrane vesicles that contain cellular components, which fuse with lysosomes to form an autophagolysosome. It is inside the autophagolysosome that the intravesicular components are degraded and, if possible, recycled by the cell (reviewed in [2 and 14]). Chaperone-mediated autophagy, finally, entails the participation of chaperones in recognizing the proteins designated for elimination by the lysosomes [14].

Autophagy is directed by *Atg* (AuTophaGy-related) genes, which are required to activate the signaling complex that triggers the formation of autophagosomes [15]. *Atg* genes were discovered in yeast, but many have orthologues in higher eukaryotes (Figure 6). Autophagosome formation entails the participation of the cytoplasmic protein LC3 (Atg8), which undergoes lipidation by phosphatidylethanolamine, and is then recruited to the nascent autophagosome membrane (Figure 7). Accumulation of lipidated LC3 protein (known as LC3-II) is used as a marker of autophagy [16].

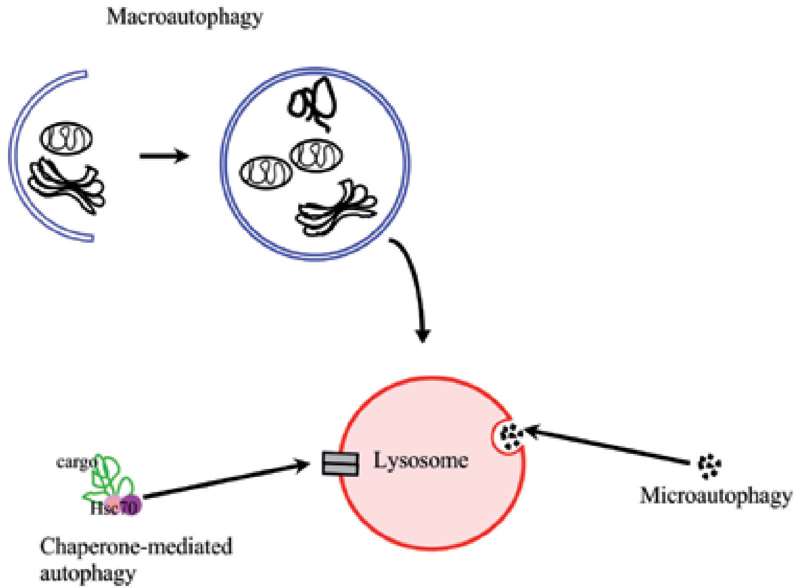


**Figure 5.** Schematic representations of the different mechanisms of autophagy. Macroautophagy – autophagy- implies the formation of a double-membrane vesicle, which engulfs cytoplasmic content that will be conducted to the lysosome to be degraded. Microautophagy is characterized by direct engulfing of cytoplasmic components by the lysosome. This process involves the remodeling of the membrane of the organelle by forming a lysosomal membrane invagination. During chaperone-mediated autophagy, the proteins to be degraded are targeted for an Hsp70, which in turn transport the target cargo to the lysosome.

Atg genes

| Nucleation | Expansion | Fusion |
|---|---|---|
| Atg 6 | Atg 1 | Vam 3 |
| Atg 9 | Atg 2 | Vam 7 |
| Atg 11 | Atg 3 | VTI 1 |
| Atg 13 | Atg 4 | YKT 6 |
| Atg 14 | Atg 5 | |
| Atg 17 | Atg 8 | |
| Vps 15 | Atg 10 | |
| Vps 34 | Atg 18 | |

**Figure 6.** Atg protein family includes more than 30 members that participate in the different events that constitute the autophagic process.

Autophagic cell death, or type II programmed cell death, is characterized by a massive engulfing of the cytoplasm by autophagic vesicles. This intense autophagic activity differs substantially from autophagy that occurs continuously at basal levels. Ultrastructural studies in Drosophila have revealed the accumulation of autophagic vacuoles in most larval tissues.

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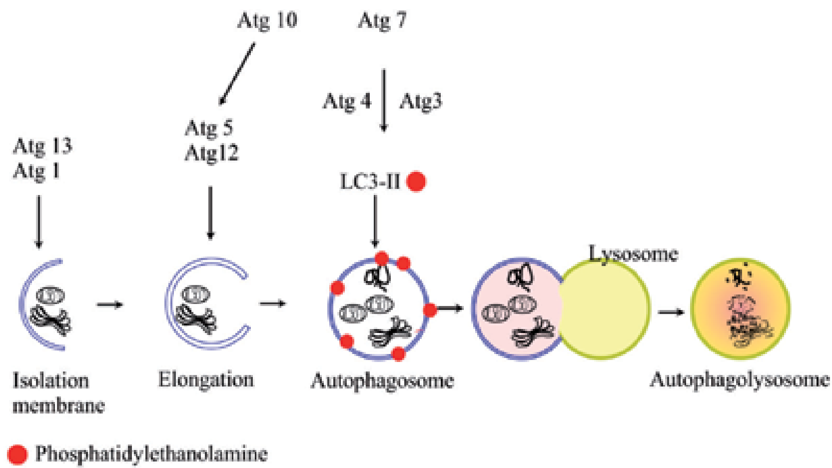


**Figure 7.** The formation of autophagic vacuoles involves the participation of different Atg proteins since the initial phases until the sequestration of cytoplasmic content. LC3 protein is lipidated by the phosphatidylethanolamine.

This type of programmed cell death begins with the degradation of cytoplasmic organelles by autophagy, though the cytoskeletal elements are conserved until the late stages of the process (reviewed in [17]).

## 3. Morphological characteristics of the necrosis process

Cell death caused by necrosis is considered an accidental, unprogrammed event that occurs under total ATP depletion [3], and that results from such external stimuli as extreme physical–chemical stress, heat, osmotic shock, mechanical stress, freezing, thawing, and high concentrations of hydrogen peroxide.

Necrotic cell death is characterized morphologically by generalized swelling of cell membranes, often accompanied by chromatin condensation and an irregular DNA degradation pattern [18]. The cytoplasmic membranes and membranous organelles dilate, and the increased cellular swelling causes the breakdown of the plasma membrane, which releases the cytoplasmic contents into the extracellular space (Figure 8). The release of the intracellular contents leads to massive cellular damage that affects neighboring cells, which explains why necrosis triggers inflammatory and autoimmune reactions. The necrosis process takes place in the absence of phagocytosis, and its final phase is characterized by the loss of the integrity of the cellular membrane. The release of the contents of necrotic cells includes molecules which act as signals that promote inflammation.

The most significant difference between programmed cell death (*i.e.*, apoptosis and autophagy) and necrosis is plasma membrane leakage and the consequent induction of inflammation in the affected tissue caused by the release of intracellular components [19].

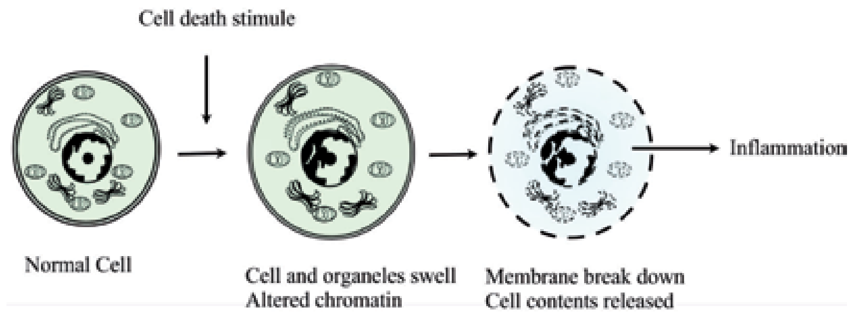


**Figure 8.** Morphological characteristics of necrosis involve the membranous swelling of the organeles, DNA degradation, and finally the release of the cytoplasmic content that affects the neighbor cells, provoking an inflammatory response.

## 4. Biochemical events during necrotic cell death

Despite these findings, however, necrosis is still considered an accidental process in which several factors exert effects on the cells to begin the elimination event. Cellular stress factors like low oxygen (hypoxia), cytokines, ischemia (restricted blood supply), heat, irradiation, pathogens, and toxin exposure can all lead to necrosis. These stimuli provoke several changes at the cellular level. While reactive oxygen species are produced by the mitochondria as a normal process, under pathological conditions, reactive oxygen molecules increase and induce damage in the biomolecules, which leads the cells toward necrosis. During necrosis, the levels of both reactive oxygen species and intracellular calcium increase (reviewed in [20]). It is important to consider that the internal cell environment is highly regulated, so certain stimuli are able to alter cell membrane permeability and thus produce an imbalance among different ions, such as potassium, sodium, and calcium. Calcium is regulated by the endoplasmic reticulum, and a loss of calcium homeostasis can lead to several intracellular alterations. In contrast, increased calcium levels can affect diverse mitochondrial functions and result in alterations of the production of reactive oxygen species. When high calcium levels are sustained over time, they disrupt mitochondrial inner membrane integrity and cause a loss of the ability to generate ATP [21] and, eventually, necrotic cell death (Figure 9). In addition to their effects inside the mitochondria, altered cytosolic calcium levels can activate different types of proteases, including calpains. Calpains are intracellular cysteine proteases present in inactive form, that may be activated by increased cytosolic calcium [22, 23]. Once activated, they can disrupt the lysosomal membrane with the resulting release of cathepsines B and L [24]. This group of reactions causes destabilization of the final membrane system. Together, these alterations cause the cell to lose its membranes such that the cellular contents are released into the extracellular space.

The molecular hallmark of necrosis is drastic ATP depletion, which is believed to be the underlying cause of cell death. There is a metabolic disruption accompanied by energy depletion and loss of ATP that leads to cellular edema, while the mitochondria become round and swollen, the endoplasmic reticulum dilates, the lysosomes are disrupted, and the formation of plasma membrane protrusions called blebs is apparent [25].

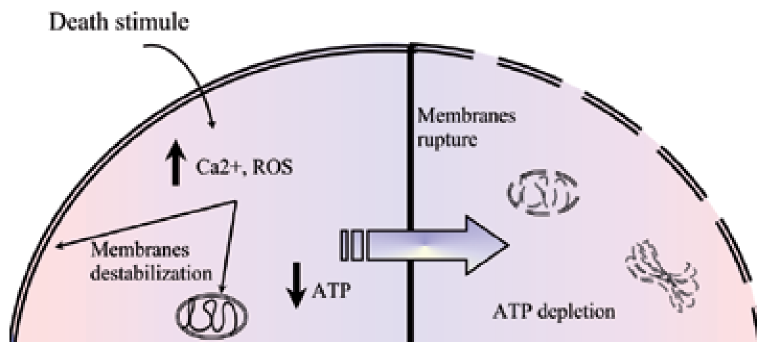


**Figure 9.** Biochemical cellular changes during necrosis cell death. The levels of both reactive oxygen species (ROS) as well as intracellular calcium (Ca2+) are increased. Sustained high calcium levels alter cell permeability of the membranes, leading to the dysfunction and ruptureof membranes. During necrosis, the ATP depletion is conducted.

Necrosis cell death occurs due to a failure in plasma membrane permeability that disrupts the plasma membrane and releases cellular components. This cell death process is associated with the indiscriminate extracellular release of soluble intracellular constituents through the permeabilized plasma membrane.

## 5. The emergence of necrosis as programmed cell death (necroptosis)

The way in which necrosis occurs allows us to determine that it is not merely accidental. Several examples of the presence of necrosis in different phases of an organism's development suggest that this process may be regulated during the embryonic stage. Necrosis is present during the longitudinal growth of bones in young animals [26], and is also found in intestinal epithelial cells in adults [27]. On the other hand, during caspase inhibition, necrosis may be activated as an alternative route of cell elimination, suggesting that it is not simply an accidental process, but that, under certain conditions could function as a programmed event called necroptosis.

The necrosis process has long been conceived as an accidental, passive event; however, recent detailed observations were able to identify dying cells by the expression of different proteins and the intracellular disposition of several proteins that may be active during the event. As mentioned above, programmed cell death, or apoptosis, can be activated by two routes, one of which entails the participation of receptors present in the cytoplasmic membrane. These receptors include TNF$\alpha$, FasL, and TRAIL that, once activated, cause recruitment of a protein complex (death-inducing signaling complex, DISC) mediated by the adaptor protein FADD (Fas-associated protein with death domain) to activate the initiator caspase-8 [28]. This activation of the caspase system triggers execution of the apoptotic process. Diverse findings indicate that the receptors involved in apoptotic cell death may also participate in the occurrence of a different type of cell death under distinct conditions of molecular resource availability. For cells that do not express caspase-8, it was predicted that they could not respond to an apoptotic stimuli directed by FasL induction. However, those results were surprising

because they included the multimerization of FADD in the absence of caspase activation. The morphology of apoptosis was not present, but ultrastructural analyses of those dying cells revealed necrotic morphological changes [29]. All these observations suggest that the receptor regulators of apoptosis were involved not only in that process, but also in the activation of a different signaling pathway that allows the formation of protein complexes which lead the cell toward a death process with necrotic features. These developments led to the emergence of a new concept of programmed cell death called necroptosis, whose morphological characteristics are similar to those of accidental necrosis, although the molecular events that occur indicate that it is a coordinated process. Necroptosis has been found under special conditions, where pro-apoptotic enzymes were absent or limited. In experimental embryo models, interdigital membrane regression in mouse embryos was effectuated by necrosis triggered by either caspase inhibition or drugs [30]. Necroptosis is thus a form of programmed cell death that has been demonstrated under experimental conditions, when apoptosis is inhibited.

## 6. Biochemical aspects of necroptosis

Several studies have succeeded in discerning the molecular events that occur during necroptosis, and it is those events that differentiate between necrosis (an accidental process) and necroptosis (a programmed process). Necroptosis has been observed in several pathological cell death events, such as ischemic brain injury, myocardial infarction, exotoxicity, and chemotherapy-induced cell death [31].

Necroptosis is morphologically characterized by several cytoplasmic changes. In fact, it is sometimes possible to distinguish the different degrees of advance of this process as the organelles swell, the cell membrane fragments, and cytoplasmic and nuclear disintegration become evident. During necroptosis, the nuclei remain intact and there is no massive caspase activation, chromatin condensation, spillage of cell contents, phagocytosis by macropinocytosis, lysosomal leakage, or oxidative bursts [32]. The term necroptosis has been introduced to identify a process of cell death with morphological characteristics distinct from those of apoptosis. Because there was no caspase activation during this process, it is called “caspase-independent”.

Necroptosis is a programmed event that ends with the delivery of the cytoplasmic contents into the extracellular space. Membrane destabilization is a consequence of different intracellular mechanisms that generate osmotic changes by damaging the ion balance. When DNA damage occurred due to reactive oxygen species, the PARP protein was activated and began the reparation process; however, this process consumed abundant ATP and that reduction initiated a sequence of events that led to a deficient cellular efflux of calcium. The decreased ATP levels affected the activity of Na+-K+ ATPase, which requires a large amount of ATP in order to function correctly. This decreased Na+-K+ ATPase activity reduced calcium release and, as a result, increased intracellular calcium levels, leading to membrane destruction (reviewed in [33]). The breakdown of the cellular membrane, in turn, released several signals

that activated the immune system. These soluble signals were proteins with pro-inflammatory properties that stimulate the recruitment of neutrophils to the site of cell death [34].

The mechanism proposed for the onset of necroptosis involves participation of the TNF-R (tumor necrosis factor-receptor), Fas, and TRAIL receptors, all of which belong to the tumor necrosis factor/nerve growth factor receptor superfamily and are involved in apoptotic cell death (Figure 10). Activation of TNF receptors by their ligands triggers different responses that involve pro-survival or pro-cell-death processes. Activated TNFR1 induces recruitment of TRADD, TRAF2/2, RIPK1, IAPs, and LUBAC to form a pro-survival complex that activates NF-kappaB, JNK, and p38 MAPKs (reviewed in [35]). However, once this complex becomes established it is able to recruit FADD and procaspase-8, which produces a complex that could initiate either apoptosis or necroptosis. Under conditions of low levels of procaspase-8, a different complex is formed, – one that includes the receptor which interacts with protein 1 (RIP1 – a serine/threonine kinase activator -) and leads to the onset of necroptosis cell death. Biochemically, necroptosis is defined as a form of cell death that is dependent on RIP1, which is the target protein in necrotic cell death induced by the TNF$\alpha$, TRAIL, and CD95 receptors [36].

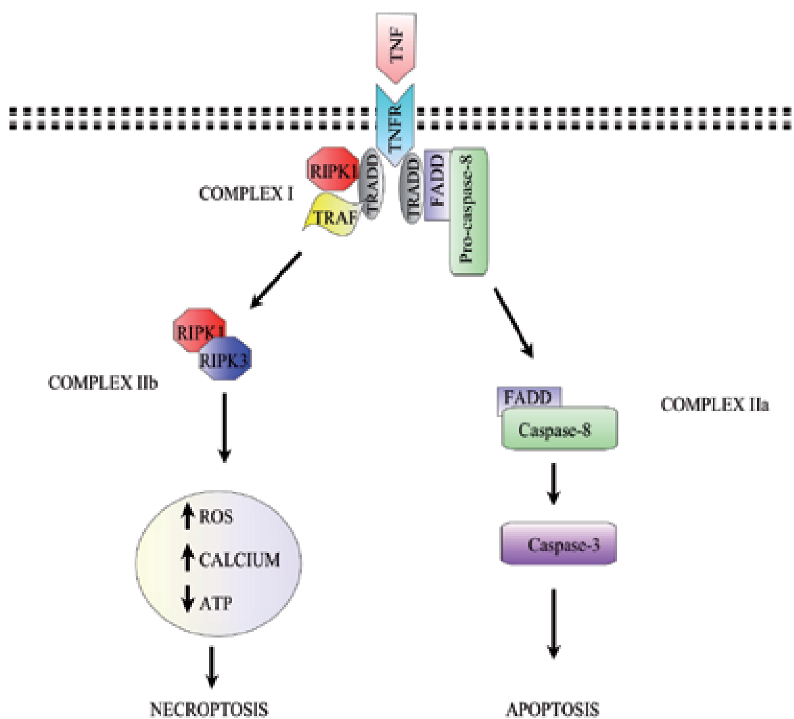


**Figure 10.** Biochemical aspects of necroptosis. TNFR recruits TRADD; this recruitment allows the formation of different complexes related to the RIPK1 protein or to the pro-caspase-8. The TRADD-FADD-pro-caspase-8 allows caspase-8 activation, which in turn activates the executer caspases, promoting the apoptosis process. Under conditions where caspase-8 is inhibited, the formation of the complex TRADD-FADD-RIPK1 initiates necroptosis.

The RIP3 is implicated in necroptosis during inflammatory responses to virus infections [37], and during cellular necrosis in response to the TNF-alpha family of death-inducing cytokines [38]. RIP3 mediates necroptosis induced by Smac mimetic and TNF$\alpha$ [38].

Activation of RIP can be directed not only by TNFR, but also by other death receptors, such as Fas. Activation of ligands associated with apoptotic cell death, such as Fas, in conditions that are unfavorable for apoptosis, that is, when caspases are absent or inhibited, allows RIP activation, which leads to death by necrosis [36, 39].

The biochemical process of necroptosis is a new and active field, so not all the routes of activation of this event have been determined. Diverse studies concur that kinase RIP is involved, as we have mentioned. Another protein, PARP-1 (poly (ADP-ribose) polymerase-1), has been shown to be involved in necrotic cell death by means of DNA-damaging agents [40], since PARP-1 is an abundant repair nuclear protein. PARP-1 is activated via TRAIL-induced necroptosis that induces ATP depletion [41]. DNA strand breaks promote the activation of PARP-1 (poly(ADP-ribose) polymerase-1) for DNA repair; PARP-1 binds to DNA strand breaks using NAD+ as substrate, generating a negatively charged PARP-1, which in turn is dissociated from DNA ends, allowing the DNA repair process [42, 43]. Intensive PARP-1 activation can generate increased NAD+ depletion and, as a result, an energy failure that leads to necrosis. In neuronal cells under severe oxidative stress, PARP-1 activation resulted in NAD + and ATP depletion that caused cell death [44].

## 7. Concluding remarks

Cell death is a normal event that controls tissue homeostasis. Today, we know that cells can be eliminated by means of different pathways that involve programmed or accidental mechanisms. Apoptotic cell death has been considered the major factor in physiological cell death, but recent evidence demonstrates that other routes of cell elimination – such as autophagy – also play important roles in maintaining homeostasis. A third route of cell death is necrosis, which was long considered an accidental form, characterized by general membrane swelling and ATP depletion. More recently, however, a new concept has been introduced: necroptosis. Necroptosis has been proposed as a kind of programmed cell death that is distinct from necrosis and apoptosis; one in which several signals involved in apoptosis participate significantly to initiate the process. It is important to note that necroptosis is an event that can be activated and regulated by such receptors as TNF or Fas – both of which are involved in the extrinsic route of apoptosis activation – when the pro-apoptotic signals are not available or are inhibited.

Activation of death receptors triggers a signaling cascade that includes activation of kinase RIP1, which in turn generates diverse intracellular reactions that lead the cell toward energy failure and conclude with the loss of intracellular homeostasis and the rupture of the membranes that, finally, generates an immunological response.

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