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Current Understanding of Apoptosis
Programmed Cell Death

Edited by Yusuf Tutar and Lütfi Tutar

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Contributors

Akinobu Ota, Yoshitaka Hosokawa, Md Wahiduzzaman, Shu-Feng Zhou, Cheng Yang, Shuo Wang, Thandavarayan Kathiresan, Chanda Siddoo-Atwal, Laila Gutierrez Kobeh, Jorge Rodríguez-González, Rosalino Vázquez-López, Arturo Alfredo Wilkins-Rodriguez, Ralf Stohwasser, Imaobong Etti, Rasedee Abdullah, Arifah Kadir

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Meet the editors

Prof. Dr. Yusuf Tutar is a faculty member at the Biochemistry Division, Faculty of Pharmacy, University of Health Sciences (Mekteb-i Tıbbiye-i Şahane), Istanbul, Turkey. He is also the head of Molecular Medicine Program. He obtained his MSc and PhD degrees from the Oregon State University and Texas Tech University, USA, respectively. He pursued his postdoctoral studies from the Rutgers University Medical School and National Institutes of Health (NIH/NIDDK), USA. His research mainly focuses on biochemistry, biophysics, genetics, and molecular biology with a specialization in the field of prion, drug design, cancer, protein structure-function, protein folding, microRNA, pseudogenes, molecular cancer, proteomics, genomics, and protein expression and characterization by spectroscopic and calorimetric methods.

Dr. Lütfi Tutar is currently an Assistant Professor at the Department of Molecular Biology and Genetics, Faculty of Art and Sciences at Kırşehir Ahi Evran University in Kırşehir, Turkey. His inter-disciplinary research focuses on bioinformatics analysis of high-throughput data, microRNAs, smallRNAs, and Heat Shock Proteins (HSPs) in human diseases and other multicellular organisms.

Contents

Preface

Apoptosis is an essential biochemical process in cell turnover, development, and chemical-induced cell death. Current knowledge and ongoing research of apoptosis highlight our understanding in designing therapeutic approaches for several diseases. This book covers four main sections: Apoptosis and necrosis, Apoptosis inducers, Proteasome and signaling pathways in apoptosis, and Radiation based apoptosis. The first section implicitly describes differences between apoptosis and necrosis processes. These two concepts were discussed on acute kidney injury. The following section elaborates small molecule induced apoptosis. Arsenic inducing apoptosis in cancer cells and more specifically artonin inducing effect in breast cancer were discussed. Further, calcium activated potassium channel link to cell survival and death was reported in this section. Then, in the third section two excellent research reports highlighted the importance of proteasome and signaling pathway: Proteasome activator PA28gamma impact on apoptosis and signalling pathways targeted by protozan parasites to modulate apoptosis and finally Apoptosis at Resistance to Chemotherapy and at Electromagnetic Radiation have been covered in the last section. Overall, the book content provides a unique perspective to the scientists and examines pathways for manipulating apoptosis. Several diseases suppress apoptosis and help cell survival therefore; the selective control of apoptosis may provide therapeutical benefits. Designing small molecules to trigger apoptosis may further augment the therapeutic effect. For these reasons, the book is a must read source for the scientist in the field.

Prof. Dr. Yusuf tutar
University of Health Sciences
Faculty of Pharmacy
Üsküdar, Istanbul, Turkey

Dr. Lütfi Tutar
Kırşehir Ahi Evran University
Kırşehir, Turkey

Apoptosis and Necrosis

From Apoptosis to Regulated Necrosis: An Evolving Understanding of Acute Kidney Injury

Shuo Wang and Cheng Yang

Additional information is available at the end of the chapter

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Abstract

Acute kidney injury (AKI) is a prevailing health threat around the world with high mortalities and heavy economic burdens. In the past, apoptosis was recognized as the main contributor to the pathogenesis of AKI. However, recent evidence has suggested that regulated necrosis also plays an important role in the pathologic process of various types of renal damages, improving our limited understanding of the complex mechanisms underlying AKI. Regulated necrosis is a newly identified type of "programed cell death" with morphologic features of necrosis, which includes necroptosis, ferroptosis, parthanatos, pyroptosis, etc. In this chapter, we summarized the molecular pathways of both apoptosis and regulated necrosis, and reviewed the potential roles and corresponding mechanisms of various cell deaths in AKI based on recent advances. We also discussed the therapeutic potentials and clinical implications based on manipulating regulated cell death. Taken together, the progress in this field lays the ground for better prevention and management of AKI in the future.

Keywords: apoptosis, regulated necrosis, necroptosis, acute kidney injury

1. Introduction

The balance between cell survival and cell death laid the foundation for any individual organisms. Cell death, especially molecularly regulated cell death, has been extensively investigated for decades in life science and medicine. Historically, cell death was roughly classified as different types: apoptosis and necrosis [1]. The term "apoptosis" derives from ancient Greek and refers to the developmentally programmed and molecularly controlled cell death, with featured morphologic changes including cell shrinkage, nuclear and cytoplasmic condensation,

DNA fragmentation, and the formation of apoptosomes [2]. Necrosis, differently, was well recognized as an accidental cell death, with unique characteristics consisting of cellular swelling, breakdown of plasma membrane integrity, and release of intracellular contents, all of which are absolutely distinct from programmed cell death [3].

Caspase-dependent apoptosis is the first identified programmed form of cell death and was regarded as the only form of regulated cell death for a very long time. However, this restricted view has been challenged with the findings of new types of regulated cell death. Exciting breakthroughs in recent years have identified a group of novel forms of cell death, with morphologic features of necrosis but molecularly controlled, termed regulated necrosis [4]. According to the recommendations of Nomenclature Committee on Cell Death (NCCD), the types of regulated necrosis are composed of necroptosis, mitochondrial permeability transition (MPT)-mediated regulated necrosis (MPT-RN), parthanatos, ferroptosis, and pyroptosis [5]. Nowadays, it is well documented that regulated necrosis broadly participates in various biological processes including organism development, immune defenses and various pathophysiological processes [4, 6]. An accumulating body of evidence has demonstrated that regulated necrosis contributes to the pathogenesis of numerous diseases and damages in different organs or tissues [4, 6, 7].

Acute kidney injury (AKI), with a heavy health burden globally, still remains a severe condition in daily clinical routines to date [8]. It is estimated that every year approximately 13.3 million people are diagnosed with AKI and AKI contributes to about 1.7 million deaths around the world per year [9]. In the past, apoptosis was supposed as the predominant form of cell demise that is responsible for renal dysfunction during AKI [10]. Much study in recent years have indicated that programmed form of cell death, no matter apoptosis or regulated necrosis, plays an important role in keeping kidney tissue homeostasis as well as contributing to the pathogenesis of AKI [11, 12]. Different methods targeting the apoptotic molecular signals have been widely explored for AKI treatment. In spite of the solid protective effects of these treatments observed in animal models, the experimental anti-apoptosis intervention strategies cannot still be translated into medical practice, which might be, at least partially, due to our ignorance of regulated necrosis in the process of AKI. An improved understanding of the pathogenesis of AKI under the view of regulated cell death might provide potential therapeutic regimens based on manipulating both apoptotic and regulated necrotic pathways.

Therefore, in this chapter, we summarized the molecular pathways of both apoptosis and regulated necrosis, and reviewed the potential roles and corresponding mechanisms of various cell deaths in AKI based on recent advances. We further discussed the therapeutic potentials and clinical implications from the clinician's underground.

2. Apoptosis in AKI

2.1. Signaling pathways of apoptosis

The classic apoptosis can be activated by the intrinsic and extrinsic pathways that both rely on the involvements of caspases [13]. To date, the signaling pathways of apoptosis

have been well delineated and for the integrity of the present review, we only emphasize on the basic and major signal events of classic apoptosis. The mitochondria are the crucial converging site for the intrinsic and extrinsic signaling [14]. And specifically, mitochondrial outer membrane permeabilization (MOMP) serves as the key to initiate the final steps of apoptosis. MOMP results in the release of pro-apoptotic components of mitochondria, including cytochrome c, AIF, etc., and thereby the activation of subsequent apoptotic executive mediators [15]. MOMP can be regulated by BCL2 family proteins consisting of pro- and anti-apoptotic factors [10]. Bax and Bak are the main members of BCL2 family and serve as promoters for MOMP; Bcl-2 and Bcl-XL, in contrast, play just the opposite role. Notably, the balance between the two parts determines the fates of cells. In the intrinsic pathway, several cellular stresses including DNA damage, intracellular calcium overload and growth factor ablation can directly induce MOMP. In response to the release of cytochrome c, apoptosome consisting of caspase 9, Apaf-1 and cytochrome c is formed in plasma, which initiates the execution of apoptosis. In the extrinsic pathway, ligation of death receptors results in the formation of DISC, which recruits adapter proteins subsequently leading to combination of caspase-8/FLIP heterodimer and caspase-8 homodimer. The homodimer then cleaves effector proteins caspases-3, -6 and -7 and thereby finishes the execution of apoptosis [16]. It is worthy to note that the extrinsic pathway is able to amplify the apoptotic signals by initiating the intrinsic pathway through caspase-8-independent activation of tBid, which can cause MOMP [14].

2.2. The contribution of apoptosis to AKI

As early as 1992, Schumer et al. have demonstrated the first evidence for the existence of apoptosis in AKI with morphologic, biochemical, and molecular detection methods [17]. Thereafter, studies about the role of apoptosis AKI and relevant potential therapeutic modalities have been under intensive investigations. Overwhelming reports exhibited that apoptosis is functionally relevant to various kinds of AKI, of which ischemia–reperfusion injury (IRI) is the most well documented [18, 19]. Besides, sepsis is another common cause of AKI, especially in the intensive care units. Studies demonstrated that apoptosis serves as an important foundation for the pathogenesis of sepsis-related AKI [20–22]. In addition to IRI and septic renal injury, toxic kidney injury is also a common clinical condition contributing to a high rate of morbidity and mortality. Cisplatin, for instance, is a widely-used chemotherapy drug with relatively high nephrotoxicity. As supposed, apoptosis plays a vital role in the pathologic process of toxic renal injury [23].

In the context of AKI, apoptotic cells are detected in different types of renal tissues within both cortical and medullary regions. The most common sites are the renal tubules, especially the proximal tubules [10]. Besides, AKI can also cause apoptosis in other renal cells, for example the vascular endothelial cells which in turn deteriorate kidney damages [24, 25].

Collectively, these studies proved the widely occurrence and definitely great functional contributions of renal apoptosis during AKI. The apoptosis was supposed as the most prominent cell death in AKI for nearly two decades, until increasing evidence shows regulated necrosis is to great extent responsible for the pathogenesis of AKI as well.

3. Necroptosis in AKI

3.1. The signaling pathways of necroptosis

Necrostatin-1 (Nec-1) was identified as a specific inhibitor of receptor-interacting protein kinase 1 (RIP1) in 2008. Since then, the molecular pathways of necroptosis have been extensively studied [26, 27]. As the best-characterized regulated necrosis, necroptosis is shown to be initiated by the engagement of death receptors, Toll-like receptors (TLRs), interferon signals, as well as intracellular stimuli from protein DNA-dependent activator of IFN regulatory factors (DAI) [28]. The details about the signaling of necroptotic cell death have been already reviewed [7, 28], and in this character, we only present the molecular pathways of TNF-α-induced necroptosis in the absence of functional caspases. Upon the binding of TNF-α to TNF receptor (TNFR)1, the adaptor molecules Fas-associated death domain (FADD) and TNF-receptor-associated death domain (TRADD) are recruited to the ligated TNFR1 successively. These adaptor molecules then bind to RIP1. Subsequently, RIP1 combines with RIP3 to assemble a complex termed "necrosome" via the interaction of RIP homotypic interaction motif (RHIM) domain on both RIP1 and RIP3 [29–32]. RIP3 goes through autophosphorylation within necrosome, which leads to the activation of RIP3 [30, 31]. Activated RIP3 subsequently recruits and phosphorylates the downstream MLKL, which is believed as the executor of necroptosis [33, 34]. The exact mechanisms underlying the execution activity of MLKL are not totally delineated yet. It is thought that phosphorylated MLKL goes through a molecular switch to translocate to the membrane and consequently disrupt the integrity of plasma membrane to finish the action of necroptosis [35, 36]. In addition, study showed that MLKL could also induce mitochondria fission via the action of phosphoglycerate mutase family member 5 (PGAM5) and dynamin-related protein 1 (Drp-1) [37]. As mitochondria play an important role in apoptosis, this result suggests a broad involvement of mitochondria in different types of cell death. But the relative contribution of mitochondria-mediated damages in the background of necroptosis needs further confirmation [38]. It is notable that other necroptotic pathways mediated by TLRs, interferon signals and DAI converge on the RIP3 and share the same downstream executing pathway, indicating the indispensable role of MLKL in necroptosis.

3.2. The contribution of necroptosis to AKI

In 2012, Linkermann and colleagues found the protective effect of Nec-1, the first-generation of necrostatins, in a murine model of renal IRI, providing the first evidence of the presence of necroptosis in AKI [39]. In this study, Nec-1 was shown to prevent ischemic kidneys from renal dysfunction and tissue damage, indicating both functional and histological relevance of necroptosis in the pathogenesis of AKI. The pan-caspase inhibitor zVAD that was used to inhibit apoptosis in treatment of kidney diseases, surprisingly, was demonstrated nonprotective in the same research. The reasons of this conflict result compared with a previous report that demonstrated the protective effect of zVAD in the context of IRI need further investigations [40]. The different methods adopted in these two independent researches may partially explain the incontinence of the therapeutic effects of zVAD. First, different clamping

time of the renal pedicles in these studies might result in the alterations of the magnitude of apoptotic and necroptotic cell death, which subsequently leads to a changed treatment effect. In addition, the time courses of the zVAD administration were also different. The profiles of cellular death kidneys will continuously evolve during AKI and apoptosis does not occur immediately after the onset of ischemia [10]. Therefore, application of zVAD just 15 min before ischemia might diminish its therapeutic effect. In accordance with this research, the protection of Nec-1 in rat and human renal tubular epithelia cells (TECs) against ischemic insults was confirmed by other investigators *in vitro* [41, 42]. Importantly, more convincing evidence of necroptosis in renal IRI was provided by using the *Rip3*-knockout mice in the following study. Linkermann et al. exhibited that *Rip3*-knockout mice improved kidney damages in contrast to wild-type mice, and Nec-1 administration in *Rip3*-knockout mice could not lead to addictive protection [43].

Besides IRI, accumulating evidence demonstrated necroptosis also contributes to AKI induced by nephrotoxic agents, including cisplatin, cyclosporin A (CsA) and imaging contrast. Tristao et al. found that Nec-1 can provide additional protection against the cisplatin-associated damage on human renal TECs on the basis of zVAD treatment [44]. Later, Linkermann [43] and Xu [45] further provided more reliable evidence to confirm the role of necroptosis in cisplatin-induced AKI with *Rip3*- and *Mlkl*-knockout mice models respectively. Ouyang et al. showed that rat TECs subjected to CsA, a widely used immunosuppressive drug for organ transplantation and other autoimmune diseases, were effectively protected by Nec-1 and knockdown of *Rip3*, indicating a role of necroptosis in the pathologic process of CsA-related AKI [46]. Furthermore, Nec-1 was similarly shown to prevent from contrast-induced AKI in a following study [47].

The existence of necroptosis was also indicated in a glycerol-induced rhabdomyolysis model [48]. The authors found that treatment with Nec-1 led to a reduced tubular necrosis, underscoring the importance of TNF-alpha-mediated tubular necroptosis in this model [48].

Taken together, these findings demonstrated that necroptosis is of vital importance for the pathogenesis of various types of AKI, suggesting a potential therapeutic checkpoint which invite further investigations basically and clinically.

4. Other regulated necrosis in AKI

4.1. MPT-RN

Mitochondrial permeability transition (MPT) mediated regulated necrosis (MPT-RN) is featured by the opening of a trans-mitochondrial membrane pore, namely the MPT pore (MPTP) [49]. CypD is identified as a controller of MPTP, which promotes the opening of this channel [50]. CypD interacts with another regulator the F_0F_1ATP synthase that maintains the inactivation of MPTP. Although the upstream pathways that initiate MPT-RN and the exact mechanisms to modulate the activity of CypD and F0F1ATP synthase remain elusive, it is believed that the opening of MPTP is capable to result in translocation of NAD^+ to cytosol and mitochondrial potential disruption [51]. NAD^+ along with ATP can be further consumed in the

process of NAD$^+$ glycohydrolases. The final result of these physiopathological alterations is the occurrence of regulated necrosis. Therapeutically, MPT-RN can be inhibited by sanglifehrin A and cyclosporin A [52].

Several independent groups of investigators have demonstrated the role of MPT-RN in AKI by detecting the contribution of CypD in the pathogenesis of kidney injuries. In 2009, Devalaraja-Narashimha et al. found that renal function, as well as the magnitude of erythrocyte trapping, tubular cell necrosis, tubular dilatation, and neutrophil infiltration in kidney histology improved significantly in CypD-deficient mice in the background of renal ischemia–reperfusion injury compared with wild-type mice [53]. Later, Hu et al. showed that knockdown of CypD by RNA interference could also protects rats from renal IRI [54]. The protective effects of CypD inhibition against kidney IRI *in vitro* and *in vivo* were further confirmed by Park et al. using a mouse model null for *Ppif*, the gene encoding CyPD [55]. Linkermann et al. evaluated CypD-deficient mice and RIP3-deficient mice in renal IRI and found that RIP3 deletion seemed to offer a better protection, providing a direct comparison between the selective contributions of MPT-RN and necroptosis to renal IRI [43]. More importantly, the researchers also showed CypD-RIPK3 double-knockout or combined application of Nec-1 and sanglifehrin A were more protective than inhibiting either of these two genes alone, indicating the coexistence of independent regulated necrosis in the same physiopathologic process.

4.2. Parthanatos

Parthanatos is the poly(ADP-ribose) polymerase 1 (PARP1)-dependent regulated necrosis [56]. PARPs cause the poly(ADP-ribosyl)ation (PARylation) of target proteins and thereby regulate various cellular bioactivities [57]. Different stimuli such as DNA breaks and Ca^{2+} signaling can activate PARP1, which induces the accumulation of PAR polymers. Both PARP1 and PAR polymers are able to delete NAD$^+$ and ATP via their PARylation [58, 59].

Increasing body of researches have demonstrated that parthanatos plays an important role in the pathogenesis of various types of AKI. By using genetic knockout models or chemical inhibitors of PARP1, several studies provided direct evidence that PARP1-dependent parthanatos was functionally related to renal IRI and showed that inhibition of PARP1 could effectively improve renal injuries [60–62]. Besides *in vivo* models, upregulated PARP1 were also detected in cultured renal tubular epithelial cells that were subjected to H$_2$O$_2$ [60, 63]. In addition to renal ischemic injury, parthanatos also contributes to AKI induced by various nephrotoxic agents [64–66]. Furthermore, the contribution of parthanatos to AKI was determined in a LPS-induced sepsis-related kidney injury model [67–69]. Taken together, these studies exhibit that parthanatos is an important participant in different forms of AKI, indicating a promising therapeutic target in clinical routines.

4.3. Ferroptosis

Ferroptosis was discovered during a pharmacological intervention in highly resistant RAS-transformed tumor cells with application of erastin by Dixon et al. [70]. Erastin, a lethal small molecule, was originally screened to eliminate cancer cells and was found to cause an unrecognized

type cell death that were distinct from either apoptosis, necroptosis, or other known regulated necrosis. This form of cell death, characterized by perioxidation, relies on accessible intracellular iron, and is therefore named as ferroptosis (ferro, ferrous ion) [70]. Erastin is believed to inhibit the system Xc- cystine/glutamate antiporter, which plays a key role in the exchange of extracellular cystine and intracellular glutamate. Cystine is required for synthesizing glutathione (GSH). Glutathione peroxidase 4 (GPX4) is an indispensable enzyme maintaining intracellular homeostasis by prevent reactive oxygen species accumulation and lipid peroxidation. Importantly, GPX4 is determined as a key inhibitor of ferroptosis, and its function is dependent on intracellular levels of GSH [71]. Therefore, inhibition of the system Xc- cystine/glutamate antiporter could result in a catastrophic decrease of GSH and thereby functional ablation of GPX4. More details about the emerging signaling of ferroptosis are provided by Yang and Stockwell [72]. Upon the introduction of ferroptosis, Dixon and Stockwell also identified a small molecule—ferrostatin-1—as an inhibitor of ferroptosis, namely the first-generation of ferrostatins, which serves as a crucial tool in the research of ferroptosis thereafter [70]. Due to the pharmacological instability of ferrostatin-1, second- and third-generation ferrostatins have been developed with promising therapeutic outlooks.

In 2014, three different teams from German and the United States reported that ferroptosis served as a crucial participant in the pathologic process of renal injuries. Friedmann Angeli and colleagues used inducible Gpx4 knockout mice to exhibit that deletion of Gpx4 led to ferroptosis-related acute renal failure and associated death. They also confirmed that Gpx4 ablation could cause extra-mitochondrial lipid peroxidation which thereby triggered ferroptosis. Furthermore, Liproxstatin-1, a spiroquinoxalinamine derivative, was demonstrated to inhibit ferroptosis *in vitro* and *in vivo* [73]. Rhabdomyolysis, as a severe and common clinical condition, is regarded as one of the main causes of AKI and rhabdomyolysis-induced AKI accounts for ~10% of all AKI cases. Rhabdomyolysis is the disruption of striped muscle followed by massive releases of intracellular molecules, in particular electrolytes and myoglobin, which induces oxidative damages and cell death. Skouta et al. subjected freshly isolated mouse kidney proximal tubules to an *ex vivo* model of rhabdomyolysis-induced AKI with or without classic ferroptosis inhibitor Ferrostatin-1 and found that Ferrostatin-1 could effectively prevent cell death [74]. Linkermann et al. have found that ferroptosis contributed to the synchronized necrosis of freshly isolated renal tubules in the context of IRI and oxalate crystal-induced acute kidney injury and Ferrostatin-1could alleviate the synchronized necrosis. Linkermann and colleagues also developed a third generation ferrostatin 16–86 with a more stable biochemical and metabolic feature, which were able to protect mice from severe IRI [75]. These reports provide direct evidence for the vital importance of ferroptosis in the pathogenesis of several types of AKI, indicating a potential therapeutic checkpoint in treating renal diseases.

4.4. Pyroptosis

Pyroptosis was initially referred to a certain kind of highly inflammatory cell death of infected macrophages [76]. Later, the cellular profile of pyroptosis has expanded from macrophages to other cell types. It is notable that a distinct feature of pyroptosis is the active release of IL-1b and IL-18 during pyroptotic cell death process, which contributes greatly to the high immunogenicity of pyroptosis [77]. Although the signaling pathway of pyroptosis, especially

the execution mechanisms, still remains elusive now, it has been documented that pyroptotic cell death results from caspase 1-dependent formation of transmembrane channels and subsequent osmotic pressure disruption [78]. In addition to caspase 1, caspase 11 are further identified as another crucial mediator of pyroptosis [79, 80]. Pyroptosis can be suppressed by chemical inhibitors VX-740, VX-765 as well as virus-derived molecule cytokine response modifier A (CrmA) [4, 76]. Few researches have focused on pyroptosis in the context of AKI. Yang et al. found that the expressions of pyroptosis-associated markers caspase 1 and caspase 11 were both significantly upregulated in a rat model of IRI and pyroptosis could also be observed in an *in vitro* model of hypoxia-reoxygenation, suggesting the existence of pyroptosis in kidney IRI [81]. Additionally, the authors demonstrated a possible regulation of endoplasmic reticulum (ER) stress on pyroptosis. But this interesting report provided no direct evidence for the functional responsibility of pyroptosis in renal injuries. The underlying physiological and pathological relevance of pyroptosis in kidneys, therefore, still remains unclear and needs intensive investigations urgently in the future [82].

5. Therapeutic implications

5.1. Combination therapy

Anti-apoptosis-based therapeutic strategies have been intensively explored for the treatment of AKI prior to the recognition of regulated necrosis. However, few anti-apoptosis interventions have been widely applied in clinical practice, despite the promising results obtained in animal models, which might be, at least partially, ascribed to our limited understanding of the regulated cell death in the context of AKI.

Thanks to the improved interpretation of the roles of regulated necrosis in kidney diseases, it is now possible to manipulate the apoptotic and regulated necrotic signaling simultaneously. In an interesting study, Tristao et al. found that the combined use of apoptosis and necroptosis inhibitors could provide additional protection in AKI, suggesting that the combination therapy targeting apoptosis and regulated necrosis might provide optimized therapeutic alternatives [44]. Moreover, combined inhibition of different regulated necrosis is also effective. Linkermann et al. demonstrated that the third-generation ferroptosis inhibitor 16–86 was able to further enhance the protective effect on IRI via combined treatment with necrostatins and MPT-RN inhibitor [75]. These results indicate that the combined blocking of several different regulated cell deaths hold the great promise to improve the current treatment for AKI. But further investigations are still needed.

5.2. Screening for next-generation inhibitors

Searching for chemical inhibitors or generating novel compounds targeting the critical checkpoints makes it possible to manipulate regulated cell death more efficiently. Compared with apoptosis whose inhibitors have been widely explored, the inhibitors of regulated necrosis warrant more explorations in the future. For example, Nec-1 is the first-generation of necrostatins that initially identified as the RIP1 inhibitor, and later widely used as a tool to distinguish necroptosis. However, a recent study demonstrated that Nec-1 was not a specific inhibitor against necroptosis

because Nec-1 could also protect $Rip1^{-/-}$ cells from ferroptosis, indicating an inhibitory effect of Nec-1 on ferroptosis [73]. More seriously, unexpected side effects of Nec-1 were observed on renal peritubular diameters [47], and on the action of indolamin-2, 3-dioxygenase (IDO) [83]. Besides, a relatively short half-life period of Nec-1 also hampers its final clinical application [83]. Thus, Nec-1 is a typical example of the original edition of regulated necrosis inhibitors that are prevailingly nonspecific and pharmacologically unstable. Great efforts have been made to searching for more effective and reliable inhibitors and a series of new inhibitors have been reported recently [34, 73, 75, 83–91]. It is remarkable that some researchers performed screens in the FDA-approved agent pools to identify effective drugs to suppress necroptosis, providing a helpful screening strategy [84, 87]. Considering that FDA-approved drugs have already been carefully evaluated in critical procedures before the clinical application, their pharmacological features and side effects are well documented. Most "new" inhibitors have not been extensively evaluated and therefore need elaborate investigations in the near future. It is indeed exciting that increasing agents targeting regulated cell death have entered clinical trials for the treatment of AKI or other kidney diseases.

5.3. Paradigm shift of cell death

There has been an interesting finding published previously that the application of zVAD, a pan-caspase inhibitor, could shift the paradigm of cell death from apoptosis to necroptosis [43]. Researches have demonstrated that apoptosis and regulated necrosis could crosstalk at various molecular levels and therefore could mutually impact each other in some certain conditions. Therefore, researchers and clinicians should be cautious about the unwanted effect in designing cell death inhibition strategies. On the other side, however, it is also reasonable to consider whether the cell death paradigm shifting is a feasible therapeutic modality in AKI treatment. Theoretically, regulated necrosis, unlike apoptosis, can cause the massive release of DAMPs and are thus more inflammatory. Manipulating the cell death profile in favor of reducing structural and functional loss of individuals may provide an optimized treatment effect. This hypothesis, of course, warrants further investigations in the following studies.

6. Conclusions

Taken together, the programmed forms of cell death in AKI consist of apoptosis as well as regulated necrosis that both serve as crucial contributors in renal injuries. An updated and better understanding of the underlying mechanism of regulated cell death provides potential "checkpoints" for AKI treatment. Therapeutic regimens, targeting the regulated cell death, warrant intensive investigations in the near future.

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

Author details

Shuo Wang[2] and Cheng Yang[1,3]*

*Address all correspondence to: esuperyc@163.com

1 Department of Urology, Zhongshan Hospital, Fudan University, Shanghai, China

2 Department of Urology, Qilu Hospital of Shandong University, Jinan, China

3 Shanghai Key Laboratory of Organ Transplantation, Shanghai, China

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Apoptosis Inducers

Apoptosis-Inducing Effect of Artonin E in Breast Cancer

Imaobong Etti, Rasedee Abdullah and Arifah Kadir

Additional information is available at the end of the chapter

Abstract

Breast cancer is among the principal cause of cancer fatalities in women. Notwithstanding the use of chemotherapy and advances in drug delivery techniques, cancer-related morbidity and mortality are still increasing with the increase in resistance to known therapeutics. This dilemma has drastically reduced the rate of survival from this deadly disease, creating a dire need for new drugs, especially from natural sources that would exhibit similar or better anticancer properties while imparting minimal adverse side effects. Among the bewildering hallmark capabilities of cancer is the evasion of apoptosis while in its course to immortality. Thus, induction of apoptosis in cancerous cells is an important breakthrough that cannot be ignored. There are, however, two major pathways of apoptosis in mammals, viz., the death receptor–mediated pathway (extrinsic pathway) and the mitochondrial-mediated pathway (intrinsic pathway). Artonin E, a prenylated flavonoid isolated from the stem bark of *Artocarpus elasticus*, was seen to induce apoptosis in MCF-7 breast cancer cells, hence halting the breast cancer cells in their journey to immortality.

Keywords: Artonin E, breast cancer, apoptosis, MCF-7 cells

1. Introduction

Breast cancers, like other forms of cancer, possess the ability for uncontrolled proliferation while resisting cell death [1]. This undue proliferation is a consequence of apoptosis evasion as well as loss of normal cell cycle control. Apoptosis is an efficient and uniquely regulated mode of cell death, involving the interplay of many factors. In mammals, there are two major pathways of apoptosis viz., the death receptor mediated pathway (extrinsic pathway) and

the mitochondrial mediated pathway (intrinsic pathway). Caspases are the key regulatory proteins for both pathways [2]. The extrinsic pathway requires the binding of ligands such as tumor necrosis factor α (TNFα), Fas ligand (Fas-L) and TNF-related apoptosis-inducing ligand (TRAIL) to the death receptor on cell surfaces and corresponding transduction of signals leading to apoptosis [3]. The intrinsic pathway, on the other hand, is mediated by the release of cytochrome c by the mitochondrial following DNA damage. The released cytochrome c then induces the formation of apoptosomes composed of apoptotic protease activating factor 1 (Apaf-1), procaspase 9, and either ATP or dATP [4]. Caspase 9 is a downstream factor that activates the executioner caspase-3, which cleaves substrates such as Poly (ADP-ribose) polymerase (PARP) [5]. The activated caspase 3 then initiates the caspase cascade that culminates in the demolition of the cell [6].

In this study, the colorimetric microculture tetrazolium assay [7] was employed to access the cells viability while assays including morphology studies, Annexin V-FITC Assay and DNA fragmentation were thereafter utilized to ascertain the mode of the cell death.

2. Materials and methods

The chemical and reagents used in the study were; MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (PhytoTechnology Laboratories, USA), DMSO (dimethylsulfoxide) (Fisher Scientific, UK), Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute medium (RPMI 1640) powders with L-glutamine (GIBCO, New Zealand), fetal bovine serum (FBS), horse serum, trypsin–EDTA, phosphate-buffered saline (PBS) tablets and penicillin (10,000 U/mL)–streptomycin (10 mg/mL) (Sigma-Aldrich, USA), acridine orange (AO) and propidium iodide (PI), annexin V-FITC (BD Pharmingen), and gel red nucleic acid stain and apoptotic DNA ladder detection kit (Abcam, USA).

2.1. Cell culture

The cell culture procedures were carried out in an aseptic condition in a Class II biohazard cabinet according to good cell culture practice (GCCP) guidelines. The MCF-7 breast cancer cell line (ATCC, USA) were grown in 25 cm^2 tissue culture flasks (TPP, Switzerland) and incubated in an incubator (Binder, Germany) at 37°C under a humidified atmosphere of 5% CO_2. The cells were maintained in RPMI supplemented with 10% FBS and 1% penicillin–streptomycin. Upon reaching about 80% confluency, the cells were washed twice with PBS and 1 mL of trypsin–EDTA solution was added to detach the monolayer cells. The trypsin was inactivated by the addition of the complete cell growth medium and the cells were collected by centrifugation at 1000 rpm (Hettich Universal 32 R centrifuge, DJB Labcare Ltd., UK) for 5 min before discarding the medium. The cell count was determined using a hemocytometer counting chamber (Marienfeld, Germany) and about 0.5 to 1×10^6 cells were subcultured into a fresh 25 cm^2 tissue culture flask containing 6 ml of fresh medium at the subcultivation ratio of 1:4. The cultures were incubated at 37°C under 5% CO_2 and 95% air.

2.2. Cryogenic preservation and recovery

The cells were preserved in liquid nitrogen to avoid loss of their original characteristics. Briefly, the cells growing in the exponential phase were subcultured. Following detachment and centrifugation, the cells were resuspended in freezing medium containing 90% FBS and 10% DMSO, as a cryoprotective agent, to yield a final cell density of 2 to 5×10^6 cells/mL. Subsequently, 1 mL of cell suspension was transferred into each labeled cryotube and allowed to stand at −20°C for 2 hours and at −80°C overnight before storage in liquid nitrogen (−196°C) (CBS Cryosystem). When needed, the cells were recovered by thawing with gentle agitation in water bath at 37°C for approximately 2 min. The cell suspension was transferred into a sterile 15 mL centrifuge tube (TPP, Switzerland) containing prewarmed complete growth medium in a biosafety cabinet and centrifuged at 1000 rpm (Hettich Universal 32 R centrifuge, DJB Labcare Ltd., UK) for 5 min at room temperature to remove the cryoprotective agent (DMSO). Finally, the cell pellets were resuspended in culture media and transferred to a 25cm^2 culture flask (TPP, Switzerland), incubated at 37°C in a CO_2 incubator. After 2 days, the medium was replaced with fresh complete growth medium.

2.3. Plating

At about 80% confluency, the cells were collected and the concentration was determined using a hemocytometer. Briefly, 0.5×10^5 cells/mL were dispensed into each well of a 96-well flat bottom tissue culture plate. To obtain the desired plating concentration of 0.5×10^5 cells/mL, the initial cell concentration was adjusted with culture medium using the following formula:

$$M_1 V_1 \ = \ M_2 V_2 \qquad\qquad (1)$$

where M_1 is the initial cell concentration, V_1 is the initial cell suspension volume, M_2 is the final cell concentration, and V_2 is the final cell suspension volume.

Using a multichannel pipette, 100 μL of the cell suspension was dispensed into each well, except the blank wells, which received 200 μL of culture medium. The cell-seeded plate was incubated overnight at 37°C to facilitate attachment.

2.4. Preparation of the test agents

Artonin E was kindly donated by Dr. Najihah Hashim of the Department of Pharmacy, Faculty of Medicine, University of Malaya. The compound was isolated from *Artocarpus elasticus*, characterized and identified as reported by [8]. The standard agents, Tamoxifen and Paclitaxel were purchase from Sigma Aldrich, St. Louis, MO, USA. These agents were dissolved in DMSO and normal saline respectively and then diluted with respective medium with highest final DMSO concentration of 0.1% for *in vitro* cell culture studies. They were diluted serially using culture medium to obtain concentrations in the range of 1.56–100 μM. About 100 μL of each concentration of Artonin E, Tamoxifen and Paclitaxel solutions was added into appropriate wells in four replicates.

2.5. Microculture tetrazolium assay

The colorimetric microculture tetrazolium assay ([7] was used to assess breast cancer cell viability. The tetrazolium dye is converted into an insoluble formazan by the action of nicotinamide adenine dinucleotide hydrogenase present in metabolically active cells. This cellular conversion into a purple-colored formazan is directly proportional to the number of viable cells. Briefly, exponentially growing cells were seeded into a 96-well flat bottom tissue culture plate at a density of approximately 0.5×10^4 cells/well and allowed to adhere to the plate by incubating at 37°C under 5% CO_2 and 95% air overnight. Following cell attachment, the cells were incubated with the tested compounds at concentrations ranging from 1.56 to 100 µM. Control cells were treated with 0.01% of DMSO, which was equivalent to the amount of DMSO used as vehicle. After each of the 24, 48 and 72 h treatment time period, 20 µL of 5 mg/mL of MTT solution was added to each well and the plate was reincubated for 4 h to facilitate catalysis by mitochondrial dehydrogenases. Next, 100 µL DMSO was added to each well to solubilize the formazan crystals. The absorbances of the resultant solutions were determined colorimetrically at 570 nm. The experiment was performed in triplicate.

We conducted a nonlinear regression analysis and utilized the GraphPad Prism software to fit a dose-response curve. The concentration of the compound that triggered a 50% growth inhibition was indicated at different time interval in **Figure 1**. The percentage viability used in fitting the dose-response curve was calculated using the following formula:

$$\% \text{ of cell viability } = \frac{A_T}{A_C} \times 100 \tag{2}$$
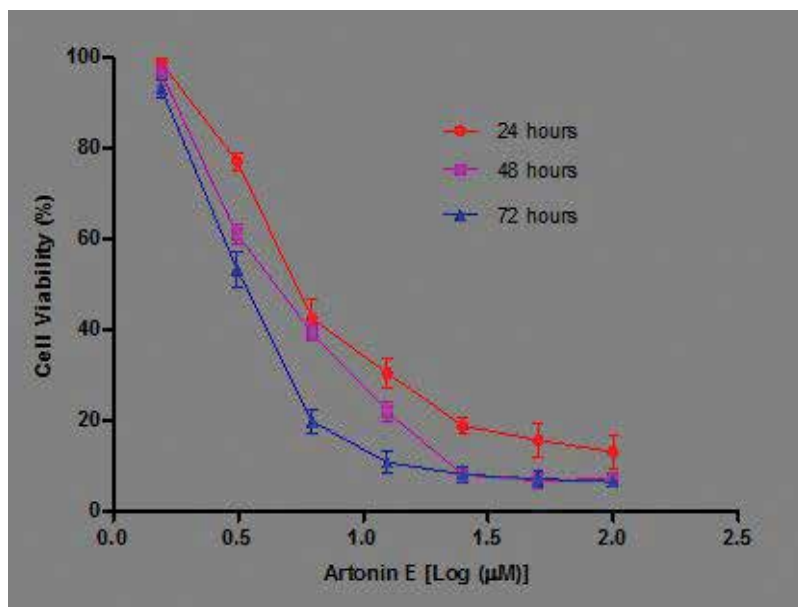
Figure 1. Viability of MCF-7 cell line treated with Artonin E. The half maximum growth inhibitory concentrations (IC_{50}) were: 6.90, 5.10 and 3.77 µM Artonin E at 24, 48 and 72 h, respectively.

Where the absorbance reading of treated samples is indicated as A_T while the absorbance of control samples treated with 0.01% of DMSO equivalent to the concentration used in dissolving the tested agent is depicted as A_C.

2.6. Cell morphological study

Acridine Orange (AO) and propidium iodide (PI) double staining assay was employed to determine the effect of Artonin E in the induction of breast cancer cell death. A total of 3×10^5 MCF-7 breast cancer cells were seeded in a six-well plate and exposed to various treatment concentrations of Artonin E (1.56–100 μM) at varying time points (24–72 h). The adherent MCF-7 cells were trypsinized, and centrifuged at 2000 rpm (Hettich Universal 32 R centrifuge, DJB Labcare Ltd., UK) for 5 min. The pellet was washed with ice-cold PBS, re-centrifuged before suspending in 20 μL of PBS. The cells were thereafter stained on ice with 20 μL dye containing 10 μg/mL of AO with 10 μg/mL of PI. Carl Zeiss Axioskop plus-2 fluorescence microscope was used to visualize aliquots of 20 μL of the cell suspension. Three fields each containing at least 200 cells were immediately assessed for viability, early and late apoptosis as well as necrosis [9]. The experiment was performed in triplicate.

2.7. Annexin V-FITC assay

Apoptosis was also investigated by detection of externalized phosphatidylserine using the Annexin V Kit (BD Pharmingen, USA). The cell preparation was carried out in accordance with the manufacturer's protocol. The prepared cells were finally subjected to flow cytometric analysis using laser emitting excitation light at 488 nm and a BD flow cytometer equipped with an Argon laser (Cyan ADP, DAKO, Glostrup, Denmark). The data were analyzed using the Summit V4.3 software.

2.8. DNA fragmentation analysis

Qualitative DNA fragmentation (Roche) kit was used in this analysis. Briefly, after trypsinization, 1×10^6 cells were collected and washed twice with PBS. The cells were then pelleted by centrifugation at 3000 rpm for 5 min, the supernatant removed and replaced with 200 μL of fresh PBS. The sample was thereafter lysed with 200 μL binding/lysis buffer consisting of 6 M guanidine-HCl, 10 mM urea, 10 mM Tris–HCl, 20% Triton X-100 (v/v), pH 4.4 and incubated for 10 min at 15–25°C. After the incubation, 100 μL of isopropanol was added and the mixture was filtered through a high pure spin filter tube combined with a clean collection tube and centrifuged at 8000 rpm for 1 min. The flow-through was discarded and the filter tube was combined again with the collection tube before washing with 500 μL of washing buffer consisting of 20 mM NaCl, 2 mM Tris–HCl, pH 7.5 in 20 mL with the addition of 80 mL ethanol. This washing step was done twice before spinning the tube dry at 13,000 rpm (Eppendorf 5424 microcentrifuge, USA) for 10 s to remove any residual washing buffer.

The extracted DNA was eluted into a fresh collection tube with 200 μL of prewarmed (70°C) elution buffer (10 mM Tris) and recentrifuged at 8000 rpm for 1 min. The quality of the

extracted DNA was assessed with a nanodrop spectrophotometer (nanodrop lite spectro-photometer, Thermo Scientific, USA). The DNA sample was mixed with 1× loading dye and electrophoresed on 1% agarose gel 75 V for 1 h and stained with GelRed™ nucleic acid gel stain. The fragmented DNA was visualized under UV transilluminator and photographed with a chemiluminescence image analyzer system (Chemi-Smart, Vilber Lourmat, Germany).

2.9. Statistical analysis

All data collected were analyzed using the GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). One way analyses of variance was performed, followed by Turkey's *post ho*c tests to compare replicate means of treatment and control groups. The significance was set at $p < 0.05$.

3. Results

3.1. Growth inhibitory effect of Artonin E on MCF-7 breast cancer cells

Artonin E, at concentrations of 6.9, 5.10 and 3.77 μM induced a half maximal growth inhibitory effect on estrogen receptor-positive breast cancer cells (**Figure 1**) at 24, 48 and 72 h respectively.

3.2. Artonin E treated MCF-7 breast cancer cells displayed morphology of apoptosis

The results of the AO and PI double staining analysis showed that Artonin E treated breast cancer cells displayed morphological features typical of apoptosis. These features included chromatin condensation and membrane blebbing. The control breast cancer cells which were not exposed to Artonin E had their normal nuclear structure displayed as green fluorescence. Early apoptotic cells were observed as bright green fluorescence resulting from the interposition of acridine orange with the fragmented DNA whereas late stage apoptosis (**Figure** 2**C** and **D**) was observed as reddish-orange, resulting from the binding of propidium iodine to denatured DNA. The apoptosis inducing effect of Artonin E was observed to be time and concentration dependent.

Challenging the cancer cells with Artonin E caused a significant ($p < 0.05$) loss in the proportion of viable MCF-7 breast cancer cells (**Figure 3**) from 94.5% to 67, 38, and 21.5% after treatment with 3, 10, and 30 μM Artonin E respectively. There was no significant ($p \geq 0.05$) increase in necrotic breast cancer cells except after exposure to 30 μM of Artonin E. This percentage decreased. There was a concentration and time dependent significant ($p < 0.05$) increase in the proportion of cells that entered the apoptotic phase.

3.3. Annexin V-FITC assay flowcytometric analysis

The externalized phosphatidyl serine was detected with Annexin V-FITC dye as an indication of apoptosis induced upon treatment of breast cancer cells with Artonin E. From the results, there was a significant ($p < 0.05$) shift in the population of the breast cancer cells from viability
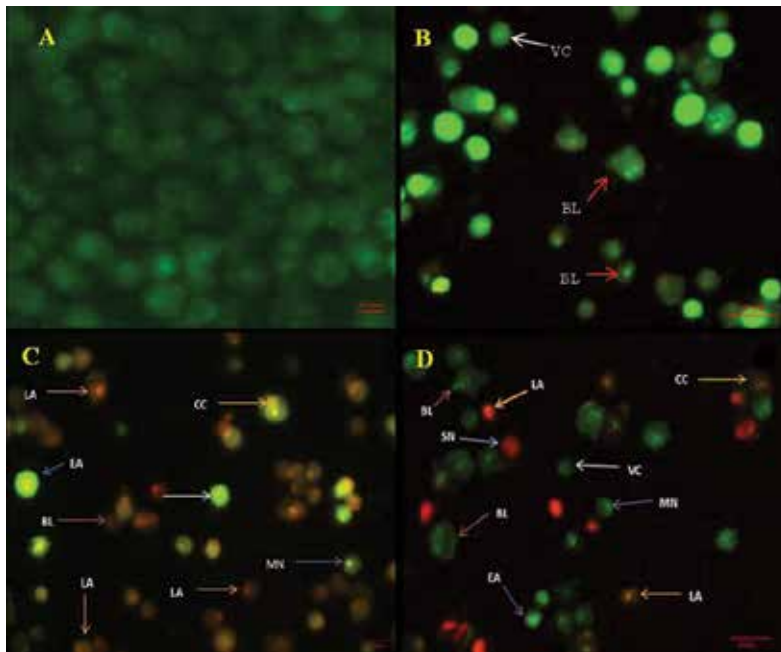
Figure 2. Acridine orange/propidium iodide double staining of MCF-7 cells after 24 h exposure. (A) Control, (B) 3, (C) 10, (D) 30 μM Artonin E. VC = viable cells; BL = cell membrane blebbing; CC = chromatin condensation; EA = early apoptosis; LA = late apoptosis; MN arginated nuclear chromatin; SN secondary necrosis. Magnification: 200×.
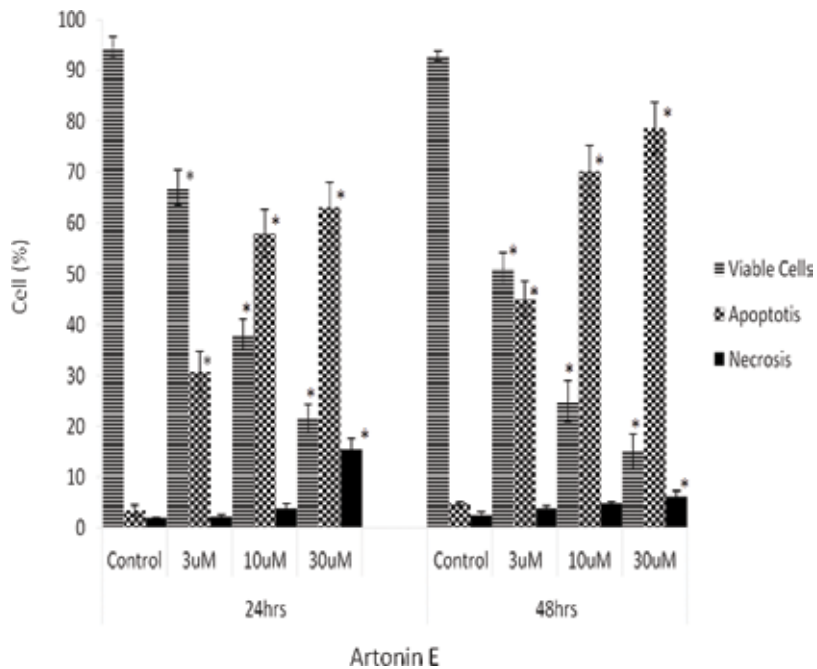
Figure 3. Quantification of early and late apoptotic MCF-7 cells after AO/PI double staining analysis. All values are mean ± standard deviation. *at each time point, means significantly different for control at $p < 0.05$.
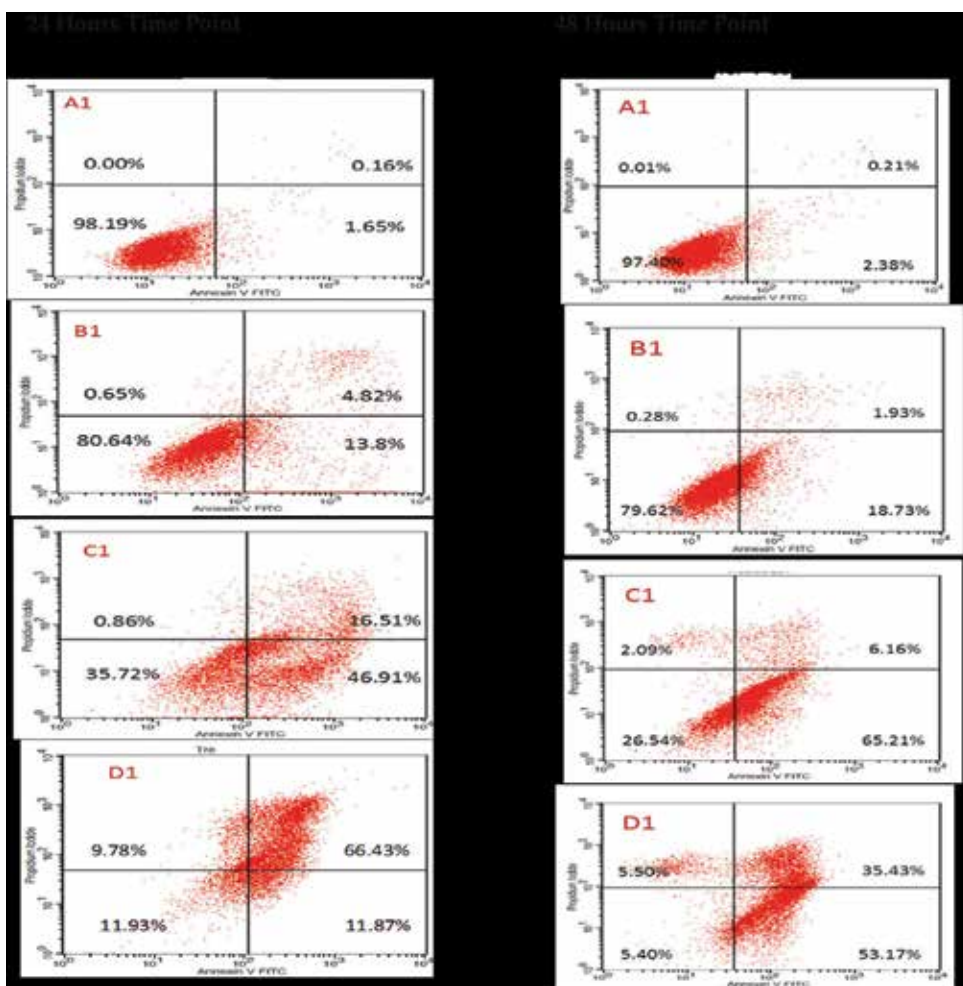
Figure 4. Representative histogram analysis of the Annexin V assay in MCF-7 cells treatment with Artonin E at 24 and 48 h. Viable cells population are shown in the lower left quadrant, while those undergoing early apoptosis are in the lower right quadrant. The upper right quadrant shows cells at late stage of apoptosis, and in upper left corner, cells at the necrotic stage are shown.

to late apoptosis (**Figure 4**). This effect was concentration as well as time dependent. After 24 h exposure of the cancer cells to Artonin E, the percentage viability decreased from 97.78% to 81.27, 36.33 and 12.0 6% when treated with 3, 10 and 30 µM of Artonin E respectively.

3.4. DNA fragmentation analysis

Another unique feature of apoptosis is DNA fragmentation which can be visualized by agarose gel electrophoresis [10]. From the results, nuclear DNA fragmentation occurred after treatment with Artonin E. This was observed in the breast cancer cells (**Figure 5**) in comparison to the untreated control group which showed intact DNA.
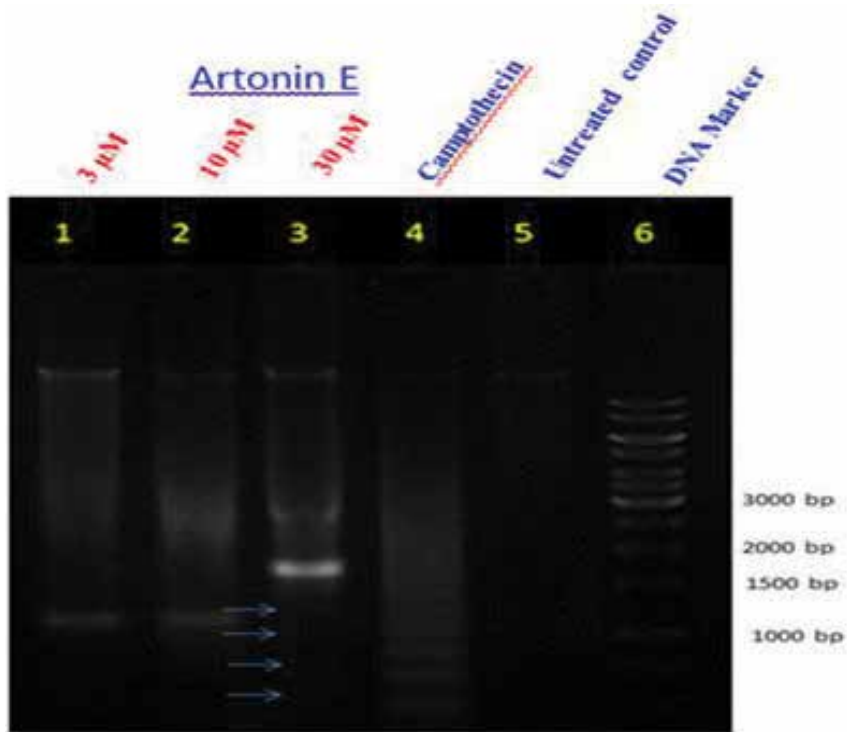
Figure 5. DNA fragmentation in MCF-7 cells treated with Artonin E after 24 h. The positive control cells were treated with 4 μg/mL camptothecin, the DNA marker is a 1 kb ladder.

4. Discussion

The death of cancer cells in a tumor is the ultimate goal of cancer drug discovery. Unlike normal cells, cancer cells possess the ability to proliferate uncontrollably while evading apoptosis. Apoptosis induction is thus a valuable characteristic of a potential anticancer drug. Hence, the cytotoxic effect of a compound can be accessed by investigating its growth inhibition on the target cancer cells. It is also vital to examine the mode of cell death induced by the compound. In this study, Artonin E, previously shown to be drug-like with a better *in silico* growth inhibitory properties when compared with similar structural analogues [11], was evaluated for its *in vitro* growth inhibition and mode of cell death induced in MCF-7 breast cancer cell line.

Artonin E was found to significantly inhibit the proliferation of this breast cancer cells in a dose and time dependent manner. At 24, 48 and 72 h, Artonin E showed half maximal inhibitory concentrations of 6.90, 5.10 and 3.77 μM, respectively. MCF-7 is estrogen receptor positive, progesterone positive and HER2 (human epidermal growth factor 2) positive and has wild type p53 [12]. Etti et al. [11] reported the *in silico* affinity of Artonin E to the human estrogen receptor α and pin pointed Artonin E as having greater binding affinity for the estrogen receptor α among the reported structural analogues from the *Artocarpus*.

It was also reported that the prenylation together with the 4^1, 5^1 vicinal diol groups in Artonin E had enhanced its affinity to the human estrogen receptor α. This affinity of Artonin E to the human estrogen receptor can be said to be mostly responsible for its better growth inhibition in MCF-7 cells observed in this study. Consistent with our findings, Obiorah et al. [13] discovered that ERα was exclusively responsible for the apoptosis induction of genistein, equol, and coumestrol, compounds which are structurally similar to Artonin E. They confirmed the phenomenon by a gene knockdown of ERα which prevented growth inhibition and apoptosis induced by these phytoestrogens. These support the involvement of the estrogen receptor in the growth inhibitory potential of Artonin E. Similarly, Turner et al. [14] also reported that prenylflavones show selectivity to estrogen receptors. Thus, it is of no doubt, that the affinity of Artonin E for the estrogen receptor is a possible basis for the observed sensitivity of MCF-7 breast cancer cells to the compound.

Various modes of cell death include apoptosis, necrosis and autophagy. However, from the results of this study, Artonin E provoked morphological features typical of apoptosis in the Artonin E treated breast cancer cells. In support of this, Carou et al. [15] and Gerl and Vaux [16], reported that apoptosis results in unique morphological changes including cell shrinkage, membrane alteration, DNA fragmentation and nuclear condensation. These features were observed in this study after treating the breast cancer cells with Artonin E. Succinctly, agents that trigger apoptosis are very essential in the management of cancer, giving that a unique hallmark of cancer cells is apoptosis evasion which is also implicated in its pathogenesis [17]. Hence, induction of apoptosis becomes a strategy for cancer drug discovery [18].

A compromise in the phospholipid membrane asymmetry of a cell, results in the externalization of phosphatidylserine. To further strengthen the assessment of the apoptotic mode of cell death, annexin V FITC in combination with a DNA binding fluorochrome, PI were utilized [9, 19, 20]. In this study, Artonin E has been seen to significantly reduce the population of viable breast cancer cells in a concentration and time dependent manner while increasing the population undergoing apoptosis. Thus, treatment of breast cancer cells with Artonin E distorted the integrity of the lipid bilayer of the cancer cells, exposing their phospholipid as detected in this study. These observations qualifies apoptosis as the mode of cell death induced by Artonin E.

Apoptotic endonucleases in the course of apoptosis, degrades chromosomal DNA into fragments [21]. This fragmented DNA can be visualized in a gel electrophoresis. In this study, after treating the cancer cells with Artonin E, its DNA was seen to have degraded into fragments in comparison to the untreated control. This DNA fragment induction by Artonin E, confirms apoptosis as the mode of cell death [22] which was also seen in the morphology of Artonin E treated breast cancer cells and the Annexin V-FITC flowcytometric assays.

5. Conclusion

Artonin E inhibited the unregulated growth of breast cancer cells and induced apoptosis in the cancer cells. It produced enhanced cytotoxicity on the estrogen receptor positive MCF-7 cells thus, halting its progression to immortality.

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

Author details

Imaobong Etti[1]* , Rasedee Abdullah[2] and Arifah Kadir[3]

*Address all correspondence to: ettiimaobong@gmail.com

1 Department of Pharmacology and Toxicology, University of Uyo, Uyo, Nigeria

2 Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University Putra Malaysia, Serdang, Malaysia

3 Department of Veterinary Preclinical Science, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Malaysia

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Arsenic-Based Anticancer-Combined Therapy: Novel Mechanism Inducing Apoptosis of Cancer Cells

Akinobu Ota, Md Wahiduzzaman and
Yoshitaka Hosokawa

Additional information is available at the end of the chapter

Abstract

Arsenic, known as both a naturally occurring toxic element and a traditionally used drug, has caught a great deal of attention from worldwide people due to its curable anticancer effect in patients with acute promyelocytic leukemia (APL). Among the arsenicals, arsenic trioxide (ATO) has been the most widely used anticancer drug. Since ATO exerts an anticancer effect by mediating apoptosis, numerous studies have made efforts to uncover the molecular mechanisms by which ATO activates and/or mediates the apoptotic signaling pathway in cancer cells. Recent advances in cancer therapeutics have led to a paradigm shift away from the traditional cytotoxic drugs toward the targeting of proteins closely associated with driving the cancer phenotype. Here, we discuss novel current arsenic-based combination therapies to treat cancer in both clinical and experimental settings. We also discuss the novel molecular mechanism underlying apoptosis induced by the combined therapies.

Keywords: arsenic trioxide, combination therapy, anticancer effect, apoptosis

1. Arsenic in cancer treatment

Arsenic, one of the ancient drugs, is currently used as a therapeutic agent worldwide because of its substantial anticancer activity. Arsenic trioxide (ATO), a trivalent arsenite (As_{III}), was initially used for the treatment of chronic myelogenous leukemia (CML) in the nineteenth century [1]. ATO readily induces apoptosis by promoting differentiation of acute promyelocytic leukemia (APL) cells and is thereby utilized as a therapeutic drug in the clinical setting [2]. ATO is currently used to treat patients with APL and recurrent/relapsed multiple myeloma (MM) [3]. Numerous studies that examined the tumor suppressive and/or proapoptotic effect

of ATO have revealed the molecular mechanism by which ATO exerts an anticancer effect in both solid cancer and hematological malignancies (see below section). Thus, accumulating evidence implicates ATO and/or other arsenicals in clinical use as a promising drug to treat cancer patients. Besides ATO monotherapy, there is a growing body of evidence that ATO may be a favorable drug when combined with not only conventional anticancer therapeutics including radiation and chemotherapy but also recently developed molecular-targeted drugs. In one example, the combined treatment of all-*trans*-retinoic acid (ATRA) with ATO has been shown to synergistically induce apoptosis of APL cells and clinically shown to be better outcome and less toxicity than the combined treatment of ATRA with chemotherapy in the treatment of patients with APL [4, 5]. This chapter summarizes the anticancer effect of ATO-based combination therapies in different types of solid cancer and hematological malignancies. Furthermore, the molecular mechanism by which ATO-based combination therapies exert a proapoptotic effect in cancer cells is discussed.

2. Novel arsenic-based combinatorial anticancer therapy

Based on the promising effect of arsenic on apoptosis in cancer cells, it has been further utilized as a combinatorial drug with other chemotherapeutic agents and/or molecular-targeted drugs to gain its anticancer effect in various types of cancer. The combination drugs, possible target molecules, molecular basis underlying combination treatment-induced apoptosis, and combination indices (CI) for each cancer are summarized in **Figure 1** and **Table 1**.

2.1. Solid cancer

2.1.1. Colon cancer

Lee et al. reported that sulindac, a nonsteroidal anti-inflammatory drug (NSAID), enhances ATO-induced apoptosis by inhibiting NF-κB activation mediated through the blocking of phosphorylation and degradation of IκB-alpha in HCT-116 cells [6]. In addition, Cai et al. reported that combined ATO-PI3K inhibitor LY294002 treatment synergistically suppresses the proliferation of colon cancer cell lines, where ATO decreases Hh pathway transcription factor Gli1 and its downstream gene expression including *BCL2* and *CCND1* [7].

2.1.2. Prostate cancer

Therapeutics in prostate cancer is based on the progression stage of the cancer, and radiation therapy is widely utilized for treatment. ATO was reported to enhance the radiation sensitivity of androgen-dependent (LNCaP) and androgen-independent (PC-3) human prostate cancer cells by mediating inhibition of the Akt/mTOR signaling pathway both in vitro and in vivo [8]. As shown in colon cancer, it has been reported that ATO inhibits the proliferation of the prostate cancer cell line PC-3 by suppressing the Hh signaling pathway and the tumor suppression effect was further enhanced by a classic Hh pathway inhibitor cyclopamine in vivo [9]. Furthermore,
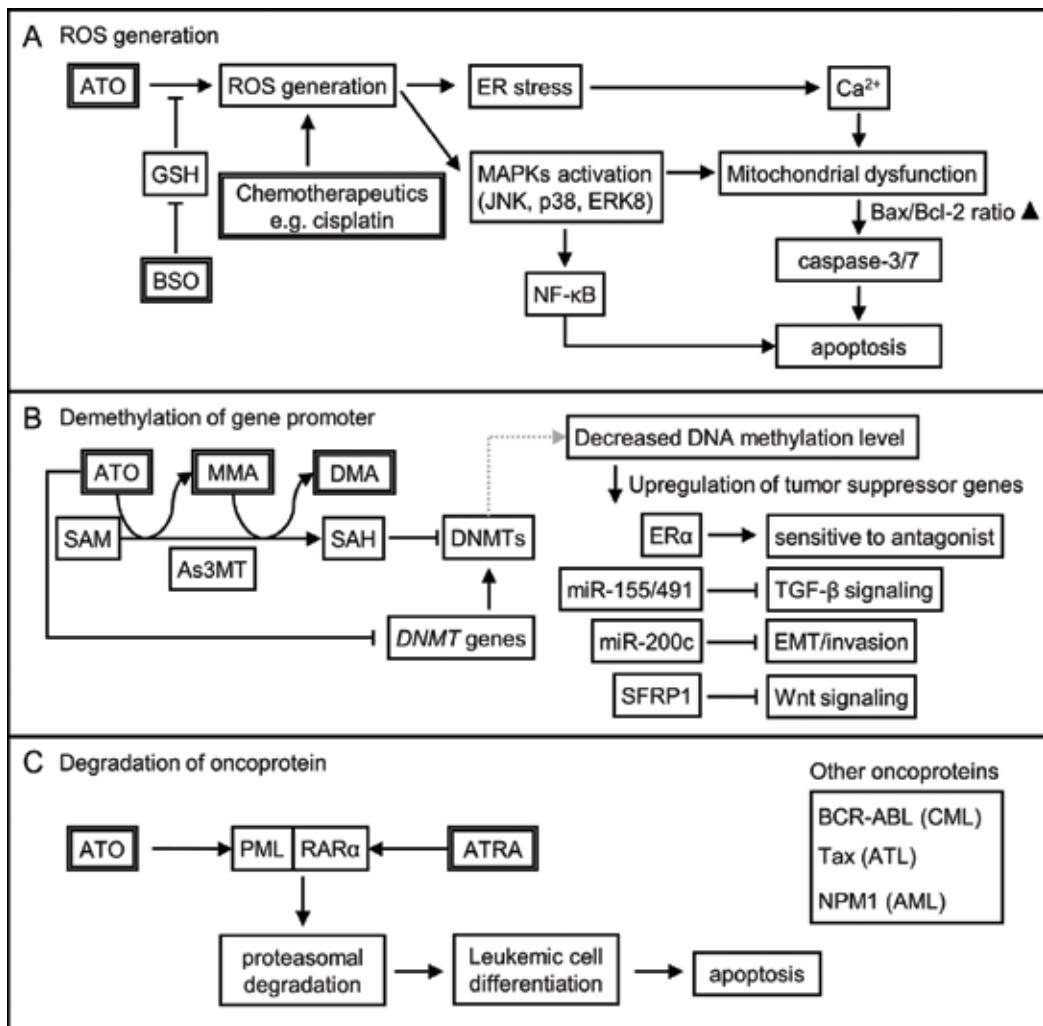
Figure 1. Molecular mechanism by which ATO exerts an anticancer effect synergistically with other therapeutics. (A) In most cancer cells, ATO generates intracellular ROS, which potentially triggers activation of the apoptotic signaling pathway. A glutathione synthesis inhibitor BSO enhances the effect of ATO-induced ROS generation by depleting GSH [43, 60], while a platinum drug CDDP cooperatively enhances ROS generation [12]. ROS-induced ER stress as well as MAPK phosphorylation can occur in mitochondrial dysfunction, which subsequently activates caspase-3/caspase-7 and induces apoptosis. (B) Arsenite methyltransferase (As3MT)-mediated metabolic methylation of ATO decreases methyl donors (*S*-adenosylmethionine (SAM)) and increases its metabolite *S*-adenosyl-L-homocysteine (SAH), which may inhibit DNA methyltransferase activity of DNMT. ATO itself downregulates gene expression of DNMTs [117]. Consequently, promoter demethylation of silenced genes, including *miR-155*, *miR-200c*, secreted frizzled-related protein-1 (*SFRP1*), and *ERα*, upregulates their gene expression, which may exert anticancer activities [22, 118–120]. The demethylation effect of ATO and other anticancer therapeutics may cooperatively induce apoptosis in cancer cells. (C) In APL cells, ATO binds to PML, while ATRA binds to RARα. Thus, combined ATO-ATRA treatment synergistically induces proteasomal degradation of PML-RARα oncoprotein, differentiation, and subsequent apoptosis [73]. ATO also can induce proteasomal degradation of oncoproteins including chimeric protein BCR-ABL (generated in CML cells), Tax (HTLV-I-encoded protein), and NPM1 (frequently mutated in AML cells). ATO and combination agents are indicated in the Double Square. MMA, monomethylarsonous acid; DMA, dimethylarsinous acid.

Combination regime	Cancer type	CI value	Mechanism of action	Ref.
ATO + radiotherapy	Prostate cancer	—	Inhibition of Akt/mTOR signaling pathway	[8]
	Oral cancer	—	Inhibition of tumor growth, angiogenesis, and metastasis	[11]
	Cervical cancer	—	Suppression of radiation-induced MMP-9 expression, ROS generation-induced MAPKs activation, and Bax translocation	[18, 19]
	Breast cancer	—	Bcl-2/Bax ratio	[23]
	Glioma	—	Increased mitotic arrest and regulation of PI3K/Akt and ERK1/ERK2 signaling pathways	[53]
ATO + cisplatin (CDDP)	Oral cancer	0.34–0.92	ROS generation, decrease in Bcl-2 protein level, and constitutive activation of caspase-3	[12]
	Ovarian cancer	0.63–0.93	Upregulation of *BAX* and *TP53* and downregulation of *HIF1A*, *IGF1R*, *MET*, and *AR* (effects by only ATO)	[15]
	Lung cancer	0.5–0.6	Increases in Bax and decreases in Bcl-2 and clusterin	[44]
TAO (As_4O_6) + CDDP	Cervical cancer	—	Synergistic activation of caspase-3	[21]
ATO + all-trans-retinoic acid (ATRA)	Glioma	—	Suppression of cancer stem cell (CSC) properties	[61]
	Hepatoma	—	Reduced GSH level	[67]
	Lung adenocarcinoma	—		
	Breast cancer	—		
	Acute promyelocytic leukemia (APL)	—	ATO and APL differentially induce proteasomal degradation of PML-RARα	[73]
	Acute myeloid leukemia (AML)/ FLT3-ITD	—	Co-inhibition of FLT3 signaling pathways	[85]
	AML/NPM1-mutated	—	Degradation of nucleophosmin (NPM1)	[86]
	Adult T-cell leukemia (ATL)/RARα-positive	—	Degradation of HTLV-I transactivator protein (Tax)	[118]

Combination regime	Cancer type	CI value	Mechanism of action	Ref.
ATO + buthionine sulfoximine (BSO)	Ovarian cancer	—	GSH depletion, increased intracellular ROS generation, and activation of oxidative stress-related pathway	[17]
	Lung cancer	—	GSH depletion	[43]
	Glioma	—	GSH depletion	[60]
	AML	—	ROS-mediated phosphorylation of JNK and BIM_{EL} and induction of intrinsic apoptosis	[87]
	Lymphoma and leukemia	—	ROS-mediated phosphorylation of JNK and upregulation of death receptor 5	[88]
ATO + bortezomib (BOR)	Multiple myeloma	0.4–0.64	Synergistic effect of ATO/BOR with p38 inhibitor (SB203580) on Bcl-2 downregulation and apoptosis in MM cell lines	[97]
	Mantle cell lymphoma	<1.0	Inhibition of NF-κB activity, decreases in cyclin D1 and Bcl-2 expression, and decreased interaction of Mcl-1 with Bak	[114, 115]
ATO + interferon-α (IFN-α)	Chronic myelogenous leukemia (CML)	—	Suppressive activity of CML leukemia-initiating cells	[108]
	ATL	—	Degradation of HTLV-I transactivator protein (Tax)	[109, 110]
	Primary effusion lymphoma	—	Inhibition of NF-κB activity	[111]

Combination index value (CI); synergism is indicated by CI < 1.

Table 1. Summary of representative ATO-based combination strategies to treat cancer.

Tai et al. reported that combined ATO-mTOR inhibitor RAD001 (everolimus) treatment synergistically induces both apoptosis and autophagy in prostate cancer cells, where enhanced autophagic cell death was accompanied by increased Beclin1 mRNA stability as well as upregulation of ATG5-ATG12 conjugate, Beclin1, and LC3-LC2 [10]. Importantly, the study showed that ATO-RAD001 combinatorial treatment more significantly suppresses LNCaP xenograft tumor proliferation than monotherapy without enhancing weight loss [10].

2.1.3. Oral cancer (oral squamous cell carcinoma (OSCC))

OSCC is the most common head and neck neoplasm and is highly associated with poor prognosis, despite advances that have been made in diagnostic and therapeutic strategies such as surgery, chemotherapy, and radiotherapy. ATO was reported as a combinatorial drug with radiotherapy [11] and a platinum-based antineoplastic drug cisplatin (CDDP) [12], both of which

are the most standard therapies for OSCC. Since ATO/CDDP-induced apoptosis was almost completely abrogated by NAC, ROS generation may be closely associated with the tumor suppression effect (**Figure 1A**) [12]. Recent publications also implicated the therapeutic application of arsenic in the treatment for OSCC. Wang et al. reported that nicotinamide phosphoribosyltransferase (NAMPT) increases in patients with OSCC and a NAMPT inhibitor FK866 and ATO cooperatively induced apoptosis and depletes intracellular nicotinamide adenine dinucleotide levels in OSCC cell lines [13]. Tsai et al. showed that the combined ATO-dithiothreitol (DTT) treatment increases proapoptotic molecules Bax and Bak and decreases Bcl-2 and p53, which leads to a significant cell death of oral cancer cells but not the non-tumor cells [14].

2.1.4. Ovarian cancer

The rate of the mortality from ovarian cancer is highest among malignant tumors of the female genital organs. As indicated in other types of cancer, ATO was reported to exert synergistic cytotoxic effects against ovarian cancer cells when it was combined with CDDP, one of the standard chemotherapeutics for ovarian cancer, and/or mTOR inhibitor RAD001 [15, 16]. Ong et al. reported that both buthionine sulfoximine and ascorbic acid differentially enhance ATO-mediated cell killing by mediating GSH depletion and the oxidative stress-related pathway, respectively [17].

2.1.5. Cervical cancer

Radiotherapy has been generally used for the treatment of patients with cervical cancer, which is the most common cancer among females worldwide, as a monotherapy and combined therapy with chemotherapeutics, such as platinum-based drugs. The combined ATO-radiation treatment was expectably reported to exert a beneficial antitumoral effect on cervical cancer cells in vitro and/or in vivo [18, 19]. Wei et al. reported that ATO decreases radiation-accelerated lung metastases probably via suppression of radiation-induced MMP-9 expression [18]. Kang et al. reported that ATO enhances the translocation of Bax protein to mitochondria and the phosphorylation level of Bcl-2, which were accompanied by activation of MAPKs including p38 and JNK [19]. Since NAC clearly inhibits the ATO-mediated cell killing as well as MAPK activation, ROS generation may play an important role in ATO-radiation-induced apoptosis. In addition to ATO, tetraarsenic oxide (TAO, As_4O_6) was shown to potentially exert an anticancer effect on cervical cancer cells. Kim et al. reported that the combined TAO and radachlorin/photodynamic therapy cooperatively suppress the proliferation of mouse TC-1 cells both in vitro and in vivo, where tumor suppressor p53 and the inducible p21 protein increased especially in combined treated tumor cells [20]. TAO was also reported to synergistically suppress tumor growth of human cervical carcinoma cell line CaSki when it was combined with CDDP [21]. The combined TAO-CDDP therapy dramatically increased the number of apoptotic cells, as similarly observed in other types of cancer cells when ATO was combined with CDDP [12, 15].

2.1.6. Breast cancer

Breast cancer is one of the leading causes of cancer-related deaths among women worldwide. In breast cancer cells, ATO was reported to reduce the expression of DNA methyltransferase-1 (DNMT1) and to induce the expression of estrogen receptor α (ERα), whose expression has been epidemiologically recognized to increase disease-free survival and indicate an overall

better prognosis (**Figure 1B**) [22]. Subsequently, combined ATO and antiestrogen tamoxifen (TAM) therapy coordinately suppressed tumor growth of a human breast cancer cell line MDA-MB-435S both in vitro and in vivo [22]. ATO was reported to enhance ^{89}Sr radiation treatment-induced apoptosis by partly modulating the Bcl-2/Bax ratio [23]. Guilbert et al. reported that ATO suppresses rapamycin (specific mTOR inhibitor)-induced phosphorylation of both ERK and Akt (Ser473), which leads to enhancement of the anticancer effect of rapamycin in vivo [24]. Cotylenin A (CN-A), a plant growth regulator, was reported to exert a favorable antitumor effect on breast cancer cells when it was co-incubated with ATO in vitro [25]. The combined CN-A-ATO treatment decreased survivin expression and increased caspase-7 expression by partly mediating ROS generation [25]. It has been reported that melatonin, a known natural antioxidant, enhances ATO-induced apoptosis by mediating ROS generation-induced MAPK activation including p38 and JNK in human breast cancer cell lines MDA-MB-231 and SK-BR-3 [26]. They also showed that mTOR inhibitor rapamycin further enhances the ATO-melatonin-induced apoptosis [26]. In addition to inorganic arsenite ATO, its intermediate metabolites monomethylarsonous acid (MMA[III]) and dimethylarsinous acid (DMA[III]) exert more cytotoxicity toward breast cancer cells than ATO, implicating application of the arsenite-related intermediates in anticancer therapy for breast cancer [27]. The combined intermediates MMA[III] and DMA[III] cryptotanshinone (a natural quinoid diterpene isolated from *Salvia miltiorrhiza* roots) strongly induce apoptosis by mediating endoplasmic reticulum (ER) stress and/or ROS generation in MCF-7 cells [27].

2.1.7. HCC and bile duct carcinoma (cholangiocarcinoma)

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy, which is the sixth most common type of cancer worldwide. Sorafenib, a known multikinase inhibitor, can extend the survival rate of patients with advanced HCC. ATO was reported to synergize with sorafenib to inhibit the proliferation and promote the apoptosis of HCC cells by diminishing the sorafenib-induced activation of Akt and/or its downstream factors, including glycogen synthase kinase-3β, mTOR, ribosomal protein S6 kinase, and eukaryotic translation initiation factor 4E-binding protein 1 [28]. ATO was also reported to potentiate the anticancer effect of genistein [29], 3'-azido-3'-deoxythymidine (AZT) [30], oridonin [31], MDM2 inhibitor nutlin-3 [32], metformin [33–34], survivin mutant (T34A) [35], shikonin [36], and andrographolide [37] in HCC cells.

2.1.8. Lung cancer

Lung cancer is the most common type of cancer worldwide. As indicated in colon cancer, there are several reports regarding the synergistic induction of apoptosis by an NSAID sulindac and ATO in human lung cancer cell lines [38, 39]. Combined ATO-sulindac treatment induced apoptosis of human non-small cell lung cancer (NSCLC) cell line A549 by mediating the mitochondrial pathway and the NF-κB pathway [38] and by mediating p53-induced downregulation of survivin [39]. It has also been reported that combined ATO-sulindac treatment induces synergistic augmentation of cytotoxicity in both human NSCLC cell lines NCI-H157 and NCI-H1299 by mediating ROS-induced MAPK phosphorylation and via c-Jun NH_2-terminal kinase-dependent Bcl-xL phosphorylation, respectively [40, 41]. Indomethacin, a nonselective cyclooxygenase inhibitor (a structural isoform of sulindac), was also shown to enhance the ATO-induced cytotoxic effect in A549 cells by mediating activation of ERK and/or p38 MAPKs [42]. Han et al.

reported that buthionine sulfoximine (BSO), a glutathione synthesis inhibitor, enhances ATO-induced apoptosis in A549 cells, in which the apoptosis was related to the increased level of intracellular ROS (**Figure 1A**) [43]. In addition, combined ATO-CDDP treatment induced apoptosis and synergistically suppressed the proliferation of human NSCLC cell lines A549 and H460, with CI values 0.5 and 0.6, respectively, where CI <1 defines synergism [44]. Lam et al. indicated that combined treatment with ATO and a selective fibroblast growth factor receptor (FGFR) inhibitor PD173074 cooperatively suppresses tumor proliferation both in vitro and in vivo in the lung squamous cell carcinoma (SCC) cell line SK-MES-1, in which ATO-PD173074 decreased FGFR1, Akt, Src, c-Raf, and Erk, at least in part by mediating proteasomal degradation [45]. Gu et al. reported that ROS-mediated ER stress and mitochondrial dysfunction were involved in the apoptosis induced by resveratrol and ATO in A549 cells [46].

2.1.9. Gastric cancer

In gastric cancer, ATO and ABT-737, a small-molecule drug that inhibits Bcl-2/Bcl-xL anti-apoptotic molecules, cooperatively suppressed the proliferation of human gastric cancer cell lines SGC7901 and MGC-803 [47]. Although ABT-737 has low solubility and oral bioavailability, the other Bcl-2 inhibitor ABT-199 has been developed for clinical use for the treatment of hematological malignancies including chronic lymphoid leukemia (CLL) [48].

2.1.10. Urothelial carcinoma (UC)

In urothelial carcinoma, Kuo et al. reported that 2-methoxyestradiol (2-ME), an endogenous derivative of 17β-estradiol, elicits synergistic cytotoxicity of human UC cell lines NTUB1 and T24 in combination with ATO, with a CI < 1.0 [49].

2.1.11. Pancreatic cancer

In pancreatic cancer, Wang et al. reported that parthenolide (PTL), a sesquiterpene lactone from the medical herb feverfew, enhances apoptosis of human pancreatic cancer cell lines PANC-1 and BxPC-3 by mediating ROS generation and subsequent caspase activation via the mitochondrial pathway [50]. The combined ATO-PTL treatment significantly reduced tumor growth rates of PANC-1 xenografts compared with those treated with either PTL or ATO alone [50]. Another report focused on the limited efficacy of ATO on cytotoxicity in pancreatic ductal adenocarcinoma probably because of the high-cellular ROS scavenging activity. Lang et al. reported that PX-478, a hypoxia-inducible factor-1 inhibitor, robustly strengthens the anti-growth and proapoptosis effect of ATO on Panc-1 and BxPC-3 pancreatic cancer cells in vitro by mediating ROS accumulation [51].

2.1.12. Glioma and glioblastoma (GBM)

In glioma, several reports indicated the effectiveness of arsenic for combination therapy. Kim et al. reported that ATO specifically increases expression of death receptor 5 (DR5), a death receptor of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in a subset of human glioma cell lines but not in astrocytes [52]. They also showed that combined ATO and TRAIL treatment synergistically reduces the survival of glioma cells, with a CI < 1.0 [52].

ATO-radiation combination treatment enhanced autophagic effects in U118-MG cells through increased mitotic arrest and regulation of PI3K/Akt and ERK1/2 signaling pathways [53]. Similar observations were reported in human fibrosarcoma and osteosarcoma, where the combined treatment arrested their cell cycle at the G_2/M phase [54, 55]. ATO is a ROS inducer, and heme oxygenase-1 (HO-1) is known to be increased in arsenic-treated cells [56]. Liu et al. reported that HO-1 inhibition or Nrf2 knockdown significantly potentiated ATO-induced cytotoxic effects on glioma cells [57]. This result suggests that ATO-induced gene expression including the Nrf2 signaling pathway may be partly involved in the resistance of cancer cells to ATO. Dizaji et al. reported that combined treatment with ATO and silibinin, a natural polyphenolic flavonoid, synergistically induces apoptosis and inhibits invasiveness in the human GBM cell line U87MG, in which the expression of cathepsin B, uPA, MMP-2, MMP-9, membrane type 1-MMP, survivin, Bcl-2, and CA9 decreased after treatment [58]. Gülden et al. reported that combined treatment with ATO and silibinin reduces the viability of A-172 by mediating intracellular arsenic accumulation [59]. As described in lung cancer, BSO depleted cellular glutathione and acted synergistically with ATO in rat C6 astroglioma cells [60]. Karsy et al. investigated the effect of ATO and ATRA on neurosphere formation of U87MG possessing wild-type (wt) p53 and/or codon-specific p53 mutant (R273H) [61]. They found that ATO and ATRA treatment could induce apoptosis of both U87-p53 (wt) and U87-p53 (R273H) and could potently suppress CSC properties in vitro [61]. It is well known that c-Myc is also required for the maintenance of CSCs of various cancers including GBM [62]. Yoshimura et al. showed that ATO and c-Myc inhibitor 10058-F4 coordinately enhanced differentiation of GBM CSCs and regressed GBM CSC tumor growth in vivo [63]. Primon et al. reported the involvement of cathepsin L (CatL) in the ATO-induced apoptotic effect [64, 65]. They found that knockdown of CatL enhances ATO-mediated in vitro cytotoxicity and apoptosis in both the GBM cell line U87MG [58] and pilocytic astrocytoma cell line MPA58 [65].

2.1.13. Other combinations

Baumgartner et al. reported that ATO-mediated apoptosis is markedly enhanced by using the polyunsaturated fatty acid docosahexaenoic acid (DHA) in 12 different ATO-resistant solid tumor cell lines including breast, ovarian, colon, prostate, cervical, and pancreatic cancer, while there was no cytotoxicity in normal skin fibroblasts, human microvascular endothelial cells, and peripheral blood mononuclear cells derived from healthy donors [66]. Lin et al. reported that combined ATRA and ATO treatment synergistically inhibits the proliferation and induces apoptosis in human hepatoma, breast cancer, and lung cancer cells in vitro [67]. Kryeziu et al. found that combined treatment with ATO and erlotinib, a selective EGFR inhibitor, acts synergistically through accumulation of DNA damage by inhibiting EGFR-mediated DNA double-strand break repair in mesothelioma, HCC, colorectal carcinoma, osteosarcoma, thyroid carcinoma, and cervix carcinoma in vitro [68].

2.2. Hematological malignancies

2.2.1. Acute promyelocytic leukemia (APL)

Patients with newly diagnosed APL, also known as a AML M3 subtype with APL-specific chromosomal translocation t(15;17) (q22;q21) and PML/RARα chimeric fusion protein, are mostly

cured after standard ATRA with chemotherapy, while toxicity and refractoriness to the treatment are observed in some patients. Accumulating evidence shows the superiority of novel combined ATRA and ATO therapy for the treatment of patients with APL in terms of event-free survival, relapse-free survival, and less hematologic toxicity, compared to ATRA with chemotherapy [4, 5, 69–72]. The molecular basis underlying synergistic effects between ATRA and ATO has been biologically demonstrated. ATRA and ATO differentially bind PML/RARα protein, the proteasomal degradation of which readily induces terminal differentiation, and subsequent apoptosis in APL cells (**Figure 1C**) [73]. In addition to the effect of ATO on the PML moiety, ATO-induced anticancer activities including inhibition of leukemic progenitor self-replication and antiangiogenic effects might be involved in the antileukemic activity. The combined ATRA with intravenous administration of ATO has been developed to combine ATRA with oral As4S4 administration as a routine treatment option for appropriate patients [74]. Moreover, Wang et al. showed that the combination of low concentrations of As4S4 and As^{3+} enhanced degradation of the PML/RARα oncoprotein and subsequent apoptosis [75]. Other modified combination regimes have been demonstrated using in vitro experimental models [76–82]. Jung et al. reported that the Src family kinase inhibitor PP2 enhances differentiation of APL cells induced by ATRA-ATO treatment [76]. Rogers et al. reported that vitamin D3 potentiates the antitumorigenic effects of ATO in HL-60 cells (PML/RARα-negative APL cell line) by enhancing nuclear DNA fragmentation [77]. The antileukemic activity of ATO was also enhanced by the combination strategies with granulocyte-monocyte colony stimulation factor [78], a noncalcemic vitamin D analog 19-Nor-1,25(OH)2D2 [79], *N*-(beta-Elemene-13-yl) tryptophan methyl [80], a selective inhibitor of epidermal growth factor receptor (EGFR) gefitinib [81], and high-dose vitamin C (ascorbic acid) [82], all of which enhance ATO-induced differentiation and apoptosis of APL cells.

2.2.2. Acute myeloid leukemia (AML)

AML is a malignant disease of the bone marrow, where juvenile leukocytes are arrested in an early stage of differentiation. It has been reported that AML patients with FLT mutations including FLT3-internal tandem duplication (FLT3-ITD) had significantly shorter overall and disease-free survival [83]. Takahashi et al. reported that combined treatment with ATO and FLT3-specific inhibitor AG1296 synergistically induces apoptosis in FLT3-ITD-positive cells, but not in Flt3 wild-type cells [84]. The combined ATO with ATRA, a novel standard treatment for patients with APL, was shown to exert synergistic cytotoxicity against FLT3-ITD AML cells via co-inhibition of FLT3 signaling pathways [85]. In addition, ATO-ATRA was shown to induce apoptosis of NPM1-mutated AML cells by targeting nucleophosmin (NPM1) oncoprotein, whose mutation possibly represents a therapeutic target because of high frequency in >30% AML [86]. As indicated in lung cancer and glioma, BSO was shown to enhance the ATO-induced anticancer effect by mediating ROS generation in AML cells [87] and other leukemic/lymphoma cells [88], suggesting that combined ATO-BSO treatment would be one of the attractive alternative therapies for cancer treatment. It has also been reported that combined treatment with ATO and dichloroacetate [89], azacytidine [90], rapamycin [91], and aclacinomycin A [92] enhances apoptosis in AML cells. Wang et al. reported that ethacrynic acid and a derivative enhance apoptosis in ATO-treated myeloid leukemia and lymphoma cell lines; this combination treatment generates high levels of ROS, activates JNK, and subsequently decreases the protein level of antiapoptotic molecule Mcl-1 [93].

2.2.3. Multiple myeloma (MM)

ATO, melphalan, and ascorbic acid (AA) combination therapy (MAC) is a therapeutic option for patients with relapsed or refractory MM [94]. Grad et al. initially showed that clinically relevant doses of AA decrease GSH levels and potentiate ATO-mediated cell death of MM cell lines [95]. Current therapeutics for MM, such as a proteasome inhibitor, namely, bortezomib (BOR) and carfilzomib, and immunomodulatory drugs, namely, thalidomide, lenalidomide (LEN), and pomalidomide, have successfully improved the patient survival, though MM remains an incurable disease [96]. In view of the current MM therapeutics, ATO was reported to enhance the anti-myeloma cytotoxicity of BOR [97] and sensitivity of MM cells to lenalidomide (LEN) [98]. Wen et al. showed that the enhanced cytotoxicity of ATO-BOR is associated with augmented STAT3 inhibition, JNK activation, and upregulation of Bim, p21, p27, and p53 as well as downregulation of Bcl-2 [97]. Jian et al. showed that ATO upregulates cereblon, the anti-myeloma target of LEN, thus potentiating the sensitivity of MM cells [98]. The anti-myeloma activity of ATO was also enhanced by the combination strategies with a vitamin E analog Trolox [99], a specific MEK inhibitor PD325901 [100], a natural quinoid diterpene cryptotanshinone (also known as STAT3 inhibitor) [101, 102], and a phytochemical sulforaphane [103].

2.2.4. Chronic myelogenous leukemia (CML)

Recent therapeutics for CML were developed by targeting the Bcr-Abl fusion protein generated from a Philadelphia (Ph) chromosome with reciprocal translocation of chromosomes 9 and 22. Despite the advances in CML therapeutics including Bcr-Abl tyrosine kinase inhibitors (TKIs), TKI therapy can produce a subpopulation of CML cells with a Bcr-Abl gene mutation that leads to resistance to TKI therapy, which results in a poorer prognosis in 10–15% of patients with CML. Several reports indicated the efficacy of ATO-based combined therapy for CML cells [104–108]. Du et al. reported that combined treatment of ATO with imatinib, which is the first approved TKI, coordinately enhances apoptosis of CML cells by mediating intrinsic (upregulation of *BAX*), extrinsic (upregulation of *TNFR1*, *CASP8*, and *CASP10*), and ER stress-related pathways (*HSPA5* and *DDIT3*) [104]. Wang et al. also showed the additive effect of ATO-nilotinib, a second-line TKI agent, on the proliferation and differentiation of primary leukemic cells from patients with CML in blast crisis [105]. In addition, combined nilotinib-ATO treatment induces ER stress-mediated apoptosis in imatinib-resistant K562 cells by mediating JNK activation [106]. Li et al. reported that anti-miR-21 oligonucleotide sensitizes K562 cells to ATO and enhances ATO-induced apoptosis probably by mediating upregulation of programmed cell death 4 (PDCD4) [107]. In a CML mice model, combined treatment with ATO and interferon-α (IFN-α) was reported to be superior to imatinib [108] in terms of overall survival of secondary recipients, indicating that ATO-IFN-α may exhaust the activity of CML leukemia-initiating cells.

2.2.5. Other leukemia and lymphoma

As the efficacy was shown in a CML mice model [108], ATO and IFN-α synergized to induce cell cycle arrest and apoptosis in adult T-cell leukemia/lymphoma (ATL)-derived human T-cell lymphotropic virus type I (HTLV-I)-transformed cells [109, 110]. El-Sabban et al. reported that combined ATO-IFN-α treatment induces the degradation of Tax, which is the

viral transactivator protein that plays a critical role in HTLV-I-induced transformation and apoptosis resistance [110]. Similarly, the enhanced ATO-IFN-α-induced apoptosis was shown in primary effusion lymphoma [111]. Darwiche et al. showed that synergism of ATO-ATRA is especially observed in the HTLV-I-transformed cells expressing RARα protein [112]. In acute lymphoblastic leukemia (ALL), low-dose ATO sensitized glucocorticoid-resistant ALL cells to dexamethasone via an Akt-dependent pathway [113]. Jung et al. and Zhao et al. independently showed the synergistic anticancer effects of ATO with BOR in mantle cell lymphoma, which is an aggressive and highly incurable B-cell non-Hodgkin lymphoma [114, 115]. Ding et al. recently reported that combined treatment of ATO with cucurbitacin B, an effective component of the dichloromethane extraction from *Trichosanthes kirilowii maxim*, synergistically enhances apoptosis by inhibiting STAT3 phosphorylation in Burkitt's lymphoma cell lines both in vitro and in vivo [116].

3. Summary

In this chapter, we show that arsenical compounds enhance cancer cell apoptosis when combined with other anticancer therapeutics including radiation, chemotherapies, and molecular-targeted drugs. Although a number of reports have shown the anticancer effects of arsenic and have discussed the possible molecular targets of ATO in malignant cells, molecular mechanisms underlying ATO-based synergistic anticancer effects with other anticancer therapeutics remain obscure. In the past decade, next-generation sequencing (NGS) technologies have tremendously improved and have clarified the whole context of genomic alterations in cancer cells, among which phenotypic and functional heterogeneity arises within the same tumor as a consequence of genetic changes, environmental differences, and anticancer therapy [121–123]. This indicates that the possibility of targeting single molecules and/or signaling pathways as well as single cellular biological processes may generate a different malignant population of cancer cells, some of which may acquire a certain drug resistance. Therefore, novel therapeutic agents and/or strategies are required to overcome drug resistance and improve both the disease outcome and the quality of life for patients with cancer. Further understanding of the relationship between induction of apoptosis and genetic/epigenetic changes in cancer cells may contribute to improvement in selectivity for cancer treatment. Additional studies are required to understand the synergistic anticancer action regarding ATO-based combination therapeutics to develop a novel combined therapy for cancer.

Author details

Akinobu Ota*, Md Wahiduzzaman and Yoshitaka Hosokawa

*Address all correspondence to: aota@aichi-med-u.ac.jp

Department of Biochemistry, Aichi Medical University School of Medicine, Nagakute, Aichi, Japan

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The Role of Calcium-activated Potassium Channel in Mitochondria-Associated ER Membrane and Its Functional Link to Cell Survival and Death

Ackuvinas Swakin Annammal Vargheese,
Krishnan Sundar and Thandavarayan Kathiresan

Additional information is available at the end of the chapter

Abstract

The process of apoptosis is not only regulated by molecular gens but it is also regulated by cellular ionic homeostasis especially K^+ homeostasis in the cell. In the past decade, molecular mechanisms of ionic regulation of apoptosis have been extensively investigated. The ionic mechanism of apoptosis are involves Ca^{2+} influx and accumulation of intracellular Ca^{2+} is convincing evidence to excessive K^+ efflux resulting in early steps in apoptosis. The BK channels play a critical role in mediating the K^+ efflux linked with apoptotic cell shrinkage. Mitochondria-associated ER membranes (MAMs) control Ca^{2+} influx between ER and mitochondria. The BKα subunits are localized in the inner mitochondrial and ER membrane and directly interact with other BK channel associated proteins (BKAPs) like, IP3R-1, calreticulin at the ER face of the MAMs, and the molecular chaperone grp78, which bridges the IP3R-1 with voltage-dependent anion channel (VDAC-1) of the outer mitochondrial membrane (OMM). The present chapter clearly depicts that how BK channels are associated with BKAPs and how they are involved in apoptosis through regulation of K^+ efflux.

Keywords: BK channel, Ca^{2+} signaling, ER-mitochondria juxtaposition, elution of protein complexes, protein-protein interactions

1. Introduction

In cochlea, the BK channels are localized in both presynaptic and extrasynaptic zone near the apical portion of inner hair cells (IHC) and outer hair cells (OHC). The BK channels are

known to be involved in noise-induced hearing loss (NIHL) [1] through activation of Ca^{2+} induced Ca^{2+} release and ROS pathway by association of BKAPs like SOD, peroxidase, catalase and GSTμ, [2]. In addition, BK channel is known to be associated with deafness proteins like γ-actin and methylthioadenosine phosphorylase (MTAP) [3]. The molecular mechanisms that regulate the BK channel and their role in NIHL and deafness remain unclear. Therefore, understanding mechanisms of BK channel regulation and its associated proteins (BKAPs) will provide insights in understanding the problems in deafness and NIHL.

Mitochondria-associated ER membranes (MAMs) control Ca^{2+} influx between ER and mitochondria. We found that BKα subunits [2] are localized in the inner mitochondrial membrane and interacted directly with other BKAPs like, IP3R1, calreticulin at the ER face of the MAMs, and the molecular chaperone grp78, which bridges the IP3R-1 with voltage-dependent anion channel (VDAC-1) of the outer mitochondrial membrane (OMM) [4]. The BK channel is associated with all other proteins having a contribution in mitochondria-associated ER membranes. Therefore, the functional regulation of BK channel and its role in MAMs remains unclear.

The novel concept of mechanism of apoptosis is in addition to molecular genes, ionic homeostasis also induces apoptosis especially K^+ in cell [5]. The ionic mechanism of apoptosis associates the accumulation of intracellular Ca^{2+} leading to uncontrolled K^+ efflux resulting in the early steps in apoptosis [6]. The BK channels play a critical role in mediating the K^+ efflux linked with apoptotic cell shrinkage. Inhibition of BK channel with iberiotoxin dramatically reduced K^+ efflux and prevents apoptosis. Therefore, enhanced K^+ efflux is an essential mediator not only for early apoptotic cell shrinkage but also for downstream of caspase-3 activation and DNA fragmentation.

2. Structure and functions of BK channel

BKα channels are involved in regulating a diversity of physiological processes such as metabolism, signaling, phosphorylation, neurotransmitter release, and modulation of smooth muscle contractions [7]. The BKα channels are activated by the cooperative effects of two distinct stimuli, membrane depolarization, and elevation of free cytoplasmic Ca^{2+} concentration. BK channels are assembled in membrane as tetramers of pore-forming α-subunits enclosing two regions, transmembrane spanning region containing two domains. They are voltage-sensing domain (VSD), which senses membrane potential and pore-gated domain (PGD) which opens and closes to control the permeability of K^+ ions. The other region, the cytoplasmic C-terminus region comprises many protein phosphorylation sites [8] such as RCK1, RCK2, leucine zipper, heme and caveolin-binding motif and Ca^{2+} bowl that regulates PGD and permeability of K^+ ions (**Figure 1**).

The pore-forming and the C-terminus domain of the BKα subunits contain several protein kinases (cAMP-dependent PKA, PKC, cGMP-dependent PKG, c-Src) and phosphatase (**Figure 1**) binding motifs which are mainly associated with a number of interacting partners to regulate the channel gating and signaling pathways. They activate the BK channel by increasing sensitivity to intracellular Ca^{2+}. The Ca^{2+} ions are bound to the electron dense of Ca^{2+} bowl
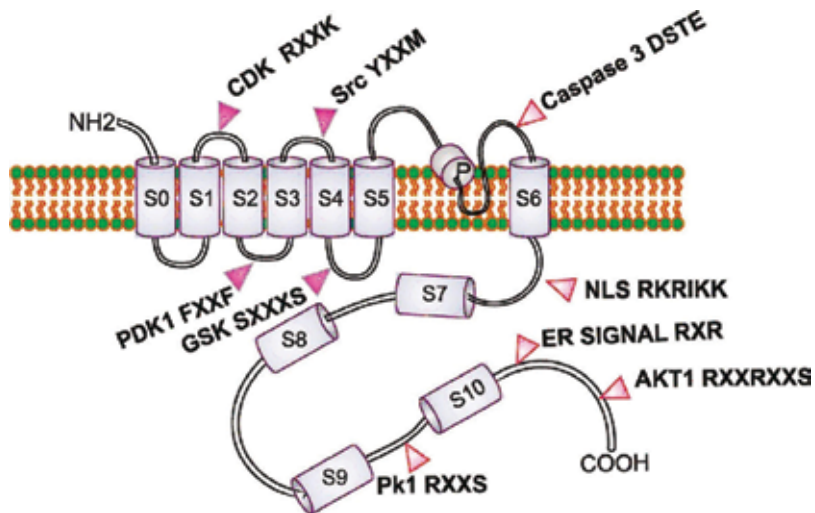
Figure 1. Significant phosphorylation sites in BKα subunit.

and activate cytosolic domain. The cytosolic domains are connected with transmembrane spanning region, S6 by 17 amino acid peptide chain, called linker peptide. A cytosolic domain through linker peptide opens and activates the gate PGD domain. Recently the cryo-EM study illustrates the BK channel structure and gating pore size is 1.7–2.0 ηm resolution [9].

The leucine zipper (LZ) motif is originally described as DNA binding proteins and reported to play an important role in both assemblies of ion channels and interactions of protein kinase and protein phosphatase. The LZ motifs serve to anchor a number of different BK channel associated proteins [10]. The LZ and EF-hand motif containing proteins regulate the mitochondrial swelling leading to apoptosis [11]. Therefore, it can be concluded that BK and other interacting proteins are regulating apoptosis through post-translational modification of phosphorylation or palmitoylation (**Figure 1**) [10].

BKα channels are sensitive to Ca^{2+} regulation through phosphorylation by serine-threonine and tyrosine kinases [12, 13]. Thirty putative phosphorylation sites were identified from seven different BKα splice variants [8]. Among them, the BK-DEC variant has an additional 60 amino acids at the extreme end of the C-terminus which contains 11 serine/threonine and tyrosine residues. The BK channels are directly involved in tyrosine phosphorylation in the presence of c-Src kinase domain in C-terminus of channel. The vital role for c-Src kinase mediating signal transduction on G-protein coupled and integrin receptor activation leads to the regulation of membrane ion channels [12]. The $\alpha5\beta1$ integrin activation leads to increasing activity of BK channel. The BK channel phosphorylation of $\alpha5\beta1$ integrin at Tyr-766 through intracellular signaling pathway involving c-Src kinase [14].

The PKC phosphorylation site (S1076) is lying on c-terminus of human BKα channel that influences the regulation of protein kinase on BKα channel activity which may subsequently alter pulmonary smooth muscle tone functions [15, 16]. This reveals the dual role of PKC on BK channel on tracheal smooth muscle. They are phosphorylation of S695 by PKC on BK

channel which is located in between the conductance of two regulators (RCK1 and RCK2) and inhibits the channel open state probability. The second phosphorylation of S1151 by PKC on C- terminus of BK channel and inhibit their channel open state activity.

3. Isolation of protein complexes from MAMs in cochlea

3.1. Maintenance of mouse cochlear hair cell culture

3.1.1. Isolation of the organ of Corti

Decapitate 2 weeks old (CBA/J) mouse at the base of the foramen magnum using scalpel. Briefly rinse the head with 70% ethanol and remove the epidermis using a scalpel blade. Open the cranium along the sagittal suture using a scalpel blade bisect the head equally half and remove the forebrain, cerebellum, and brainstem using blunt dissection. Remove the temporal bones, dip them briefly in 70% ethanol, and transferred into 35 mm dish. Remove the bulla and surrounding tissue from the petrous portion of the temporal bone and identify the conch shaped cochlea and separate it from the vestibular system using forceps. Remove the calcified bony labyrinth of the cochlea carefully removed from basal region to apical end. Spiral ligament and organ of Corti is tightly attached and coiled inside the bony labyrinth. Carefully remove the organ of Corti by securing the spiral ligament at the hook region of the base using forceps and unwinding it as you move apically. Begin at the base and remove the spiral ligament from the organ of Corti using no. 55 fine forceps.

3.1.2. Micro-isolation of hair cells from sensory epithelium of organ of Corti

The isolation of hair cells from mouse cochlea was described [17]. Remove the organ of Corti at the base of hook region by using two ½ cc insulin syringes with the help of U-100 28G½ needles as forceps. The organ of Corti consist of spiral limbus and sensory epithelium (outer and inner hair cells) cells starting from apex to base of organ of Corti. The sensory epithelium was separated from spiral limbus with help of insulin syringes. The sensory epithelium explant was transferred into fresh Petridis (35 mm) containing 1 ml of DMEM with 10% FBS, ampicillin (10 mg/ml), and 400 µl of each poly-L-ornithine (0.01%) and laminin (50 µg/mL). The Petri dish was incubated at 37°C with 5% CO_2. After 48 h carefully change the above fresh medium then the adhesive outer and inner hair cells was started multiplication on the Petri dish.

3.2. Transfection of candidate genes in mouse cochlear hair cells by nucleofector device (Lonza)

The mouse cochlear hair cell culture is washed with pre-incubated PBS buffer and adds 1 mL of trypsin solution then incubated 37°C for 1 min. and harvest (2.5 × 105) cells. The cells were centrifuged at 300 g for 10 min at room temperature and the supernatant removed and

appropriate nucleofector solution (containing 2 μg of plasmid vector or 100 ηM of SiRNA and 100 μL of P4 primary cell 4D-nucleofector X solution) added into the nucleocuvette. Gently tap the nucleocuvette vessels to make sure the samples were premixed and the cover bottom of the cuvette. Place the nucleocuvette vessels and close the lid into retainer of the 4D-nucleofector X unit and select the appropriate program [18]. After completion of the run carefully remove the nucleocuvette vessels and resuspended cells with pre-warmed culture medium. The gene expression or down-regulation will be observed after 4 h transfection to 4 days.

3.3. Mitochondria and endoplasmic reticulum isolation from mouse hair cell culture

The mitochondria were isolated from mouse cochlear hair cell cultures using a kit per manufacturer's instructions (Qproteome TM Qiagen). The cells were washed with PBS buffer and harvest these cells with 1 ml of disruption buffer containing protease inhibitor cocktail and incubated 10 min at RT. After 10 min the cells were centrifuged at 6000×g for 10 min and collect the pellet and discard the supernatant. The pellet was resuspended in purification buffer followed by spun at 20,800×g for 15 min. Mitochondria and ER were layered on the surface of a density gradient centrifugation. Both mitochondria and endoplasmic reticulum were removed from the respective gradient and diluted in storage buffer, and spun at 8000×g for 10 min. The pellet consisting of purified mitochondria and endoplasmic reticulum were either resuspended in storage buffer and store in −80C or resuspended in protein lysis buffer get mitochondria and endoplasmic reticulum proteins.

3.4. Transmission electron microscopic studies

The BKα gene cloned in pCDNA3.1 mammalian expression vector and transfected in mouse cochlear hair cell cultures by using Nucleofector device. After transfection, both control and BK transfected cells were harvested and the cells were fixed with glutaraldehyde. The fixed cells were transferred in to wire gauge. The morphological changes of hair cells with respective of apoptosis such as plasma membrane dissolution; mitochondrial bulging, ER, and nuclear fragmentation were observed under electron microscopy with different concentration of BK transfection in the absence and presence of curcumin loaded silica nanoparticles. The synthesis of silica nanoparticles and encapsulation of curcumin will be carried out using a published procedure. One of the Co-PI is familiar with the synthesis and characterization of silica nanoparticles. The silica nanoparticles will be coated with polymers (polyethylene glycol) (PEG) or polyethylenimine (PEI) to enhance the biocompatibility of the nanoparticles. Initially, the amount of BK with appropriate time intervals is evaluated to activate apoptotic pathways in mouse cochlear cells.

3.5. Proteomics approach

The appropriate BKα gene was transfected with mouse cochlear hair cell cultures. After 48–72 h transfection, the mitochondria were harvested from the control and BK transfected mouse cochlear hair cell cultures. The proteins from mitochondria were isolated from both

control and BK transfected hair cells. The 50 μg of proteins were mixed with sample buffer and loaded in IEF gel strips. IEF will be performed using 7 cm immobilized pH gradient (IPG) gel strip, pH 3–10 (Protean IEF Cell System, Bio-Rad). Proteins were resolved by IEF in the first dimension and SDS-PAGE (12% acrylamide) in the second dimension. Precision Plus (Bio-Rad) molecular weight marker was used to determine relative mobilities. Gels were stained with silver staining and images were captured using the Molecular Imager versa doc MP Imaging System (Bio-Rad). The resolution of the scanning gel was 53 μm, and images were processed with the standard version of PDQUEST software (Bio-Rad), which is used to identify spots by pi and molecular weight with the help of standards. The BKα transfected protein gel is compared with control gel and qualitative differences of appeared (up-regulated proteins) and disappeared (down-regulated proteins) protein spots were excised and subjected to reduction, alkylation, and trypsin digestion as described previously [19]. Peptides were extracted and concentrated under vacuum centrifugation. The peptides of each sample were injected into LC–MS/MS then identification of each protein spots.

4. Structural link between ER and mitochondria

The mitochondria consists of two membranes viz. an outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) which have several convoluted foldings called as cristae. The shape of mitochondria is very heterogeneous in living cells from sphere to interconnected tubules [20]. The formation of mitochondrial network is well documented by the continuous movements of mitochondria by motor proteins. During the mitochondrial movements rarely two mitochondria encounter each other to form fuse [20]. Sometimes the mitochondrial tubules can undergo fission to form two or more mitochondrial units. However, both mitochondrial processes are much complicated because of the coordination of fusion and fission of four lipid bilayers. The first mitochondrial fusion protein Fuzzy onions 1 protein (Fzolp) was identified in *Drosophila melanogaster* [21]. Later two Fzolp homologus mitofusin 1 (MFN1) and mitofusin 2 (MFN2) were identified from mammals [22]. The MFN1 and MFN2 have very high 81% homology and both are localized in the outer mitochondrial membrane [23]. These proteins are formed by the docking of two juxtaposed mitochondrial fusion through their transmembrane spanning region [24]. During the mitochondrial fission both Fis1 and dynamin-related protein1 (Drp1) play a vital role in mammals [25].

The endoplasmic reticulum is an extensive network of cisternae and microtubules and stretches from the nuclear envelop to the plasma membrane of all eukaryotic cells occupying 10% of the total cell volume [26]. The ribosomes bind to the peripheral of ER and to the nuclear envelope comprising the rough ER and ribosome-free is called as smooth ER. The peripheral ER consists of sheath-like cisternae and the thickness of the sheets and diameter of the tubules ranges from 60–100 nm. ER and microtubule associated proteins play a predominant role in shaping of mammalian cells [27]. The cytoskeleton membrane proteins 63 kDa (CLIMP63), VAP-B/Nir3 couple and p22 are involved with dynamics of ER and microtubule [28]. CLIMP63 is an integral peripheral ER membrane proteins which is anchoring of ER to microtubules and maintains the spatial distribution of ER network. The p22 has myristoylated EF-hand protein binds microtubule in a Ca^{2+} dependent manner and providing a link between

ER morphology and Ca^{2+} [29]. The ER morphology is still controversial even though reticulons and DP1 proteins are enriched in ER tubule rather than sheets and nuclear envelope [30]. Another protein dynamin-related membrane GTPases atlastins are involved in the control of morphology of ER by promoting the branching of the tubules [31].

5. Protein liaison in tethering to ER and mitochondria

Close contact between the membrane of ER and outer mitochondrial membrane was first identified in late 1960 by several independent groups [32]. ER membranes co-purifying with mitochondrial fractions were observed under electron microscopy which, revealed that direct communication between cisternal space of ER and inner mitochondrial membrane (IMM) space [33]. The 20% of mitochondrial surface were direct contact with ER and each contact appears to vary between 10 to 25 nm in length [34]. The functional importance of these two organelles contact sites is further established by the quasi-synaptic mechanism of transmission of Ca^{2+} crucial function of during apoptosis.

The nature of ER–mitochondrial tethering has remained largely elusive. Szabadkai et al. [35] reported that IP3R is localized on membrane of ER and VDAC is localized on the OMM and both are physically attached through 75 kDa glucose-regulated proteins (GRP 75) (**Figure 2**). IP3R play a major role on the mobilization of calcium from ER to mitochondria as a function of apoptosis [36]. During steady-state transfer of Ca^{2+} molecules from ER to mitochondria, the mitochondrial anti-apoptotic proteins Bcl2 is drastically reduced [37]. The phosphofurin acidic cluster sorting protein-2 (PACS)-2 is also involved in the regulation of apoptosis
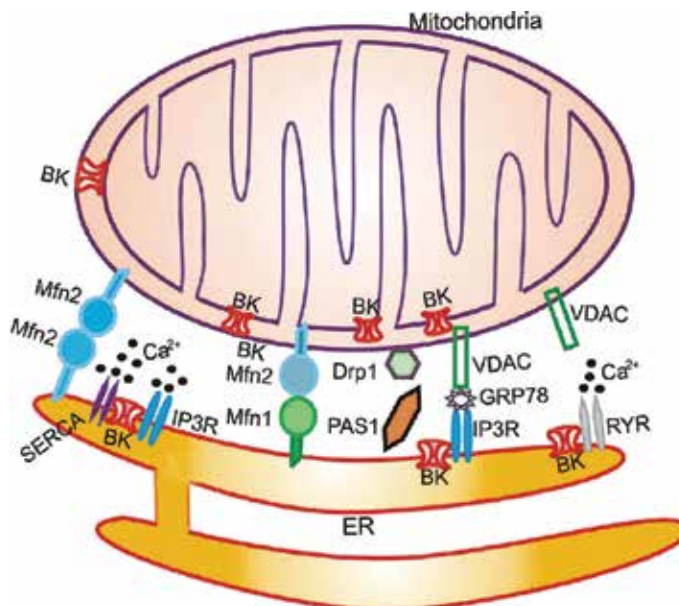
Figure 2. Tethers between ER and mitochondria.

through induces Bid translocation to mitochondria [38]. The first direct ER-mitochondrial tethering proteins are Mfn1 and Mfn2 [39]. They are localized on both organelles membrane and close contact point between the organelles. In addition, they are involved in maintaining the shape of both organelles. Moreover the regular juxtaposition of ER and mitochondria is the cytoskeletal network [40]. Both the organelles were bound with microtubules (MTs) and actin cytoskeleton that provide a scaffold that stabilizes the contact points between the compartments [41]. Recently identified mitostatin protein is binds with keratin and intermediate filaments that inhibits the juxtaposition of ER and mitochondria.

6. The role of bk channel role in mitochondria and ER interactions

Earlier, the molecular mechanism of BK channel function in mouse cochlea we studied, through the system biology approach aided by the sensitivity of coimmunoprecipitation, shotgun mass spectrometry methods to identify 174 BK channel associated proteins (BKAPs). Based on BKAPs developed transient BKα interaction networks are enriched with functional attributes of metabolism, trafficking and scaffolding, development and differentiation, signal transduction, and transport [2]. We, for the first time, employed a large number of ion channel associating proteins in the cochlea. The BKAPs were analyzed through subcellular localization. The majority of the BKAPs nearly 30 and 15% were localized in mitochondria (both membrane and matrix) and endoplasmic reticulum. Comprehensive understanding of the BK channel role in mitochondria and endoplasmic reticulum function has remained unclear. The past decade has concealed an number of unexpected protein-protein interactions that fundamentally changed our view of the localization and functional interactions of proteins inside cells. The functional role of BK channel in mitochondria is no exception.

One of our recent bioinformatics studies has revealed that the proteomics data (BKAPs) with functional attributes remarkably identified nearly 20 and 10% of novel mitochondrial and endoplasmic reticulum BKAPs involved in pro-apoptotic and anti-apoptotic properties which have not been looked in to, so far. Based on these results the preliminary experiments of tunnel assay for the BK overexpressed CHO cells that confirmed 65% cell death when compared with control. Till now, the exact mechanism behind the mitoBK and ER-BK channel role in pro-apoptosis is not known. It is expected that proposed project will give a new dimension in the biomedical field, which in turn may be useful for understanding the mechanism of hearing loss and noise-induced hearing loss (NIHL).

The Ca^{2+} activated potassium channel is playing a predominant role in mitochondria and endoplasmic reticulum-associated proteins. These proteins were contributing either K^+ influx or K^+ efflux of cells through the Ca^{2+} ion binds with Ca^{2+} bowl of c-terminus region [2]. Superoxide dismutase, glutathione S transferase μ, GAPDH, VDAC, and peroxidase are involved in ROS pathway as well as the candidate for BK channel associated proteins. The endoplasmic reticulum proteins calreticulin, GRP78, inositol triphosphate receptor (InsP3R), protein SET, VCP, HSP70, and protein disulfide isomerase are involved in the regulation of calcium-induced calcium release (CICR), protein folding and clearance. These proteins are known to BK channel associated proteins [2]. These two groups of proteins are involved in

the mitochondrial associated-ER membrane (MAMs) to regulate calcium signaling of CICR and cell death. However, we are only beginning to understand the spatial organization and interorganellar signaling in between ER and mitochondria and their functional regulations.

Mitochondria-associated ER membranes (MAMs) control Ca^{2+} influx between ER and mitochondrial subunit. We found that BKα subunits [2] localized in the inner mitochondrial membrane directly interact with other BKAPs like IP3R, calreticulin at the ER face of the MAMs. The molecular chaperone, glucose-regulated-protein 78 (grp78), is linked with inositol 1,4,5 triphosphate receptor-1 (InsP3R-1) and voltage-dependent anion channel (VDAC-1) of the outer mitochondrial membrane (**Figure 2**) [42]. Both Ca^{2+} overload and depletion of the ER Ca^{2+} pool can result in changes of two signaling pathways of unfolding protein response (UPR) and ER overload response (EOR) [43]. Latter induces mitochondrial membrane permeabilization (MMP), opening of the permeability transition pore (PTP), and release of cytochrome c and subsequent engagement of the mitochondrial apoptotic pathway [44]. The Ca^{2+}-sensitive dehydrogenases of the Krebs cycle [45] are stimulated as increased mitochondrial Ca^{2+} boosts ATP production. Increasing ATP production leads to more leakage of free electrons, causing the formation of superoxides. The resulting oxygen ions, free radicals, and peroxides are collectively called reactive oxygen species (ROS), which are effective to damage DNA and apoptosis.

ER membrane BK channels play a predominant role in ER retention, retrieval and normal trafficking [46]. InsP3R and BK channels are localized in the same hotspot region of glioma cells of lipid raft however; they are not directly interacting with each other [47]. The InsP3R are tightly linked with BK channel through continuous releasing of Ca^{2+} molecules in organelles and they are bind with EF-hand motif of Ca^{2+} bowl and activate BK channel to release the K^+ ion (K^+ efflux).

The cellular Ca^{2+} ion are transported through the plasma membrane by receptor and voltage-sensitive channels. Once inside the cell, Ca^{2+} can either interact with Ca^{2+} binding proteins or sequestered into the ER or mitochondria. Both Ca^{2+} overload and depletion of the ER Ca^{2+} pool can result in changes of protein folding and an increase of ER stress by either unfolding protein response (UPR) or ER overload response (EOR) [42]. From these two responses, both incorrectly folded and accumulated proteins are eliminated through apoptosis [48]. Ca^{2+} depleted from the ER is then taken up by the mitochondria, leading to Ca^{2+} overload in this organelle. The latter induces mitochondrial membrane permeabilization (MMP), opening of the permeability transition pore (PTP), and release of cytochrome c and subsequent engagement of the mitochondrial apoptotic pathway [44]. The increasing mitochondrial Ca^{2+} boots ATP production leading to more leakage of free electrons, which results in the formation of superoxides. These oxygen ions, free radicals, and peroxides, collectively called ROS, damage DNA and leads to apoptosis. Reactive oxygen species (ROS) play a key role in mechanism for induction of cochlear damage under various patho-physiological conditions [2]. Caspase-12 is localized in ER and activated by ER stress, including the accumulation of excess proteins that leads to cell death.

7. Conclusions

BK channel is involved in the regulation of pro-apoptosis in mouse cochlear hair cells. A group of proteins is involved in the regulation of mitochondria-associated ER membrane

(MAMs) and in the maintenance of intracellular Ca^{2+} level in cells. The MAMs associated proteins are known to be BK channel associated proteins. However, the details of interactions of MAMS associated proteins with BK channel and their mechanisms of post-translational modification such as phosphorylation and palmitoylation remain elusive. It is proposed that the present study will clearly depict how $BK\alpha$ is involved in apoptosis through K^+ efflux and regulation of BKAPs by phosphorylation. The phosphorylation of activation or inactivation of specific kinases and phosphatase binding motifs mainly regulate the channel gating and different signaling pathways. From these studies, we gain the knowledge of the mechanism of BK channel mediated apoptosis and identification of novel therapeutics to inhibit intracellular calcium induced stress-related apoptosis in cochlear hair cells. Therefore, through this study it is expected to find novel therapeutics for deafness and noise-induced hearing loss population in our society.

Acknowledgements

Conflict of interest

The authors declare that there are no conflicts of interest.

Author details

Ackuvinas Swakin Annammal Vargheese, Krishnan Sundar and Thandavarayan Kathiresan*

*Address all correspondence to: t.kathiresan@klu.ac.in

Department of Biotechnology, School of Bio and Chemical Engineering, Kalasalingam University, Krishnankoil, Virudhunagar District, Tamil Nadu, India

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Proteasome and Signalling Pathways in Apoptosis

Proteasome Activator 28γ: Impact on Survival Signaling and Apoptosis

Ralf Stohwasser

Additional information is available at the end of the chapter

Abstract

This chapter aims to explain the functional impact of the proteasome activator (PA)28γ protein in cellular survival. The mechanistic complexity of this proteasome activator, encoded by the PSME3 gene and overexpressed in tumors in correlation with the degree of severity, is attracting growing attention. Taking anti-apoptotic properties of PA28γ into account, a simple view might explain therapy resistance of tumors by the presence of high concentrations of this proteasome regulator. A more sophisticated approach would consider functional parameters such as subcellular distribution, competition with other proteasome regulators, and factors affecting heptamer assembly, proteasome binding, and activation. Recently, PA28γ has been attributed as a proteasomal recognin, particularly for intrinsically unstructured proteins (IUPs), targeted by ubiquitin-independent proteasomal protein (UIPP) degradation. Other reports demonstrated inhibitory or stimulatory effects of PA28γ on turnover of substrates of the ubiquitin- and ATP-dependent proteasome system (UPS). Since the understanding of functional implications of PA28γ on diverse signaling processes has grown exponentially and the orchestration of proteolytic systems within apoptosis is fairly complex, this article summarizes the recent developments in PA28γ biology with emphasis on cell survival signaling pathways such as DNA repair and apoptosis.

Keywords: acetylation, apoptosis, autophagy, ATM kinase, caspase, cancer, DNA double-strand break (DSB), DNA damage response (DDR), B-cell lymphoma-extra-large (BclX$_L$), PSME3 gene, proteasome activator 28 gamma (PA28γ; REGγ), proteasome activator 700 (PA700), inhibitor of apoptosis (IAP), inflammation, melanoma-associated antigen 1 (MART-1), metabolism, NF-κB signaling, nuclear body (NB), nuclear speckle (NS), Cajal body (CB), c-Myc, p53, promyelocytic leukemia (PML), sirtuin (SirT), ubiquitin (Ub)

1. Introduction

Posttranslational modification and regulated intracellular proteolysis are intimately linked with regulation of proliferation, differentiation, and apoptosis. Aforementioned survival processes depend on the steady-state levels of proteins acting as molecular switches of signaling networks and regulated proteolytic systems activating or removing such switches. Proteasomal protein degradation bifurcates into ubiquitin-dependent pathways, namely, the ubiquitin proteasome system (UPS) and ubiquitin-independent proteasomal protein (UIPP) degradation pathways [1]. Due to the modular structure of the proteasomal proteolytic system, composed of 20S proteasomes, 19S regulatory particles (RP), and 11S proteasome activator 28 (PA28) subtypes, it is challenging to identify definite relations between 20S proteasomes, regulator-associated proteasomes, and their substrates.

Among 11S regulators, proteasome activator 28γ (PA28γ) has earned remarkable attention, due to its pronounced overexpression in cancer and its functional relation to tumor biology. Several evidences indicate that PA28γ, in addition to its role as a 20S proteasome activator and as a putative substrate recognition module, might also act as a modulator of E3 ubiquitin ligases. Since the first contributions of PA28γ to UIPP have been reviewed a decade ago [2], a reevaluation of proteasome biology with a focus on the intrinsic pathway of apoptosis seems justified. Growing knowledge about the remarkable efforts of various proteasomal proteolytic systems in cell survival will improve the mechanistic knowledge for target definition and specific drug design to counterstrike cancer [3].

2. Ubiquitin proteasome system (UPS)

The discovery of the ubiquitin proteasome system seems to be a never ending story of surprise, starting with the first observations of non-lysosomal, ATP-dependent protein degradation [4], recognizing ubiquitin as a barcode label for proteins dedicated for proteasomal degradation [5] and, finally, realizing that ubiquitin labeling is not an absolute requirement for proteasomal recognition, unfolding, and decay [6].

2.1. Ubiquitin conjugation and barcode properties

The enzymatic machinery-conjugating ubiquitin (Ub) or ubiquitin-like proteins (UBLs) to lysine is based on hundreds of enzymes, activators, inhibitors, and substrate adaptors, establishing an information-based system for multiple purposes in signal transduction. Fifteen human E1 enzymes (ubiquitin-activating enzymes (UBAs)) initiate the conjugation cascade either specifically using ubiquitin or one of the ten different UBLs as substrate. Thioester-linked E1-Ub/UBL can be transferred to about 80 E2 enzymes (ubiquitin-conjugating UBC) via transesterification to the thiol group of an active site cysteine. Finally, more than 600 monomeric or multimeric E3 enzymes (ubiquitin protein ligases), either harboring RING or HECT domain motifs, constitute molecular scaffolds catalyzing the substrate-specific transfer

of thioester-linked Ub/UBL mostly to lysine residues of substrate proteins. Since ubiquitin contains seven putative conjugation sites, linear or branched Ub conjugates can be formed by isopeptide bonds creating individual barcodes. Processing of conjugates, either as a requirement for substrate channeling into proteasomes or for fine tuning the barcode information, is performed by cysteine proteases of the deubiquitinase (DUB) protein family [7]. The resulting barcodes are guiding proteins into different fate decisions, namely, affecting subcellular locations (UBL:SUMO), enzymatic activity (UBL:NEDD8), or 26S/30S proteasomal turnover (UBL: ubiquitin) [3–5, 8].

Ubiquitin-K48 conjugates are mostly used as degradation signal [5] that can be recognized by 19S regulatory particles (PA700) attached to one or both ends of 20S proteasomes, either forming 26S or 30S proteasomes, respectively [9].

2.2. 20S proteasomes

2.2.1. Core proteases of UIPP and UPS

The term "20S proteasome" encompasses variant isoforms of compartmentalized, barrel shaped 700 kDa core proteases (CP) of $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ stoichiometry [10]. Six active site β-subunits of the catalytic chamber are processed from zymogenic β1, β2, and β5 precursor subunits during standard (s20S) proteasome assembly, establishing the amino-(N)-terminal threonine nucleophiles central to the catalytic mechanism [11]. Proteasomes reveal cleavage site preferences for hydrophobic, basic, and acidic P1 residues resembling specificities of prototypic proteases (chymotrypsin-like (CHYT), trypsin-like (TRYP), caspase-like (CASP). Typically, for a compartmentalized protease, access to the active sites is regulated by gated pores, formed by the amino-terminal chains of α-ring subunits [12]. The narrow orifice formed by the α-rings restricts access of most native proteins. Therefore, unstructured protein regions or protein unfolding is a prerequisite for substrate channeling, conducted by 19S 11S proteasome activators [12–15].

Due to tissue-specific or cytokine-inducible expression of proteasomal active site subunits [16, 17], a high diversity of 20S proteasome complexes is principally available to associate with proteasome activators (PA) such as PA700 (19S), PA28$\alpha\beta$, or PA28γ (11S). As reviewed elsewhere [9, 10], proteasomes have been purified and biochemically characterized from various sources, most recently discovering the unique properties of 20S proteasomes from thymus (t20S) [18], a discovery highly relevant for the understanding of positive selection of T cells [19]. Constitutively, expressed standard 20S proteasomes (s20S) and immune proteasomes (i20S) have been compared with respect to their different assembly kinetics, half-lives, and catalytic capabilities [20].

The pro-inflammatory cytokine interferon-γ (IFN-γ) modulates the composition of the immunoproteasome (i20S), due to the expression of zymogens (pro-subunits) of inducible active site subunits (iβ1, iβ2, iβ5). During POMP-dependent proteasome assembly in proximity to the ER, active site subunits of constitutively expressed proteasomes (s20S) are replaced by the inducible subunits, forming immunoproteasomes (i20S). The i20S complexes reveal faster assembly (about 21 versus 80 min) and shorter half-lives (27 versus 133 h) and differ in

activity and cleavage site preferences from s20S proteasomes [21, 22]. In concert with other intracellular proteases, combinations of both 20S isoforms, associated with 11S/19S activator complexes, contribute uniquely to processing of peptides for MHC class I antigen presentation of self or foreign antigens [23].

2.3. 19S regulatory particle and 26S/30S proteasomes

Degradation of ub-labeled proteasome substrates depends on 26S/30S proteasomes. These protease complexes contain either one or two 19S regulatory particles (RP) associated with the α-ring subunits of s20S or i20S CPs. The 19S RP (proteasome activator PA700) is composed of base and lid sub-complexes that reveal defined pathways of chaperone-assisted assembly [9]. The base ATPase sub-complex (subunits Rpt1–Rpt6) harbors a chaperon-like unfoldase activity [24] and is involved in substrate channeling into 26S or 30S proteasomes. The lid sub-complex enables ub-conjugate binding, as well as deubiquitination for ubiquitin recycling and substrate channeling. Assembly, composition, and topology, as well as specific biological aspects of 26S/30S proteasome genesis and function, have been reviewed comprehensively by others [20, 25, 26].

2.4. Proteasome activator 28$\alpha\beta$ and MHC class I antigen presentation

The proteasome activator (PA) 28 protein family is encoded by three PSME genes. The IFNγ-inducible PSME1 and PSME2 genes accompany immunoproteasome expression. However, preferential association with i20S versus s20S proteasomes has not been supported by in vitro kinetics of proteasome activation [14]. Apart from their ability to form homo-heptameric proteasome-activating complexes in vitro, the PA28α and PA28β subunits prefer assembly into PA28$\alpha_4\beta_3$ hetero-heptamers as revealed by kinetic and structural analysis [14, 15]. PA28$\alpha_4\beta_3$ activates 20S proteasomes by channeling, regulating the width of the narrow orifice, and excluding natively folded protein substrates. The control of substrate import and export of peptide products, possibly as a molecular sieve, or due to allosteric regulation of proteasomes by PA28$\alpha_4\beta_3$ may affect proteasomal processivity and quality of peptide products [27, 28].

Double knockouts of the IFNγ-inducible members of the PSME1 and PSME2 genes [29] were examined with respect to immunological functions and immunoproteasome assembly. Substantiating the role of so-called hybrid proteasomes (PA28$\alpha_4\beta_3$-20S proteasomes) [30, 31], it was found that association of 26S proteasomes with PA28$\alpha_4\beta_3$ increased proteasomal activity and presentation of particular antigens [29]. However, PA28$\alpha\beta\gamma$-null mice showed normal antigen presentation with some antigens and impairment with others. Based on these observations, it has been stated that PA28$\alpha\beta\gamma$ is not a prerequisite for antigen processing in general, but seems to be essential for the processing of certain CTL epitopes [23].

2.5. Biochemical properties of proteasome activators 28γ

The third member of the PA28 protein family, proteasome activator (PA)28γ, encoded by the PSME3 gene, was discovered as nuclear Ki autoantigen about four decades ago [32]. Biochemical key features of the heptameric 11S regulator PA28γ [33–39] are summarized in **Table 1**.

PA28γ Protein	Cellular Context & Biochemistry	Cellular or Biochemical Activities and Experimental Evidences	Refs
Biochemical Properties	Monomer/Heptamer: Assembly into PA28γ₇ PA28γ₇-20S- PA28γ₇-UIPP Protease Activates proteasomal TRYP-like activity	Purification of 11S regulator Characterization of PA28α/β/γ PA28α/β/γ: Regulation by IFNγ Properties of nuclear PA28γ Gelfiltration/Ultracentrifugation	[33] [34] [35] [39]
Sequence/ Structure	PA28γ₇ structure not available; structural information from other members of the PA28 protein family	First human PSME3 cDNA sequence PA28α₇: first heptameric structure 11S-20S proteasome structure Structure of mammalian PA28α₃b₄	[36] [37] [38] [15]
Subcellular Localization Nuclear Dynamics	PA28γ is majorly localized to the nucleus, but cytosolic shuttling has been observed. Specific functions in nuclear bodies	Biochemical evidence: purification Immunhistochemistry PA28γ and 20S colocalize in NS CBs: PA28γ-coilin interaction DSB: ATM-dep. PA28γ-20S recruitment Maintenance of chromosome stability	[39] [50] [40] [41] [42] [71]
Phosphorylation by /Association with Protein Kinases	ATM MEKK3 CHK2	PA28γ-20S recruited to DSB sites PA28γ apparently stabilized PML and CHK2 association of PA28γ	[42] [43] [69]
Acetylation	CBP acetylase SirT1 deacetylase	K195 acetylation increases heptamer stability and 20S association	[45]
Sumorylation	PIAS1 E3	PIAS1 deficiency: proteasomal degradation of p21 impaired	[44]
PA28γ Turnover	Half-life: 24 h	Pulse chase experiment	[46]
	Caspase 3/7	PA28γ harbors DGLD cleavage site	[47]

Table 1. Cellular biochemistry of PA28γ.

Particularly, roles in nuclear dynamics [40, 41], posttranslational modification [43–45], or contributions in apoptotic signaling are emphasized [46, 47].

Application of PA28γ as a diagnostic marker in inflammatory autoimmune or neoplastic diseases has been proposed [48, 49]. Initially identified as a nuclear autoantigen [32, 39, 50],

context-dependent cytosolic presence has also been reported. In breast cancer, PA28γ accumulates in nuclei, whereas in pancreatic cancer cells, PA28γ emerges in the cytosol [51], indicating that cellular context generates diverse scenarios with respect to subcellular localization and function of PA28γ. Nuclear-cytosolic shuttling, mediated by posttranslational modification, depends on cell types and conditions [44, 52, 53]. Sumoylation of amino-terminal lysine residues K6/14/12 by PIAS1 (E3) enhances cytosolic localization and increases stability, presumably prerequisites for proteasome association and substrate recognition [44]. Contrarily, acetylation at K195 by CREB-binding protein (CBP) favors assembly of nuclear PA28γ. Deacetylation by sirtuin 1 (SirT1) has been demonstrated in mammalian cells. Since K6 and K14 residues can be acetylated as well, competition with sumoylation cannot be excluded [45]. Furthermore, phosphorylation of PA28γ by protein kinase MEKK3 resulted in a concomitant increase of PA28γ levels in Cos cells [43]. Stress kinases Chk2 and ATM have been reported to phosphorylate PA28γ, thereby affecting protein-protein interactions (PPI) and functions in DNA damage response [42].

2.6. Overexpression of PA28$_\gamma$ in cancer

Several authors showed the association of PA28γ with tumorigenic pathways and correlation of expression with malignancy and metastasis. In skin carcinogenesis, PA28γ is involved in modulating WNT signaling [54]. The improved survival properties of PA28γ overexpressing cells [46] and the well-documented overexpression in tumors and cancer cell lines raise the question, how PSME3 gene expression is regulated on the transcriptional and translational level.

PA28γ-mediated mechanisms supporting cell survival in cancer cells are of high relevance for cancer therapy, particularly in the light of profoundly increased levels of PA28γ in cancer cell lines and in tumor tissue. Highly increased steady-state level of PA28γ was observed majorly in epithelial and mesenchymal tumors such as thyroid neoplasm [55]; breast tumors, particularly those with poor prognosis [56–58]; colorectal cancers [48]; hepatocellular carcinoma [59]; and oral squamous cell carcinoma (OSCC) [60].

3. The PA28γ scaffolding hub in tumor biology

A particular interest of this article is the question, how UPS and UIPP are involved in regulating cell survival pathways intimately linked with tumor biology and therapy resistance (**Figure 1**). Cyclin-dependent protein kinases (CDKs) are one regulatory implement of proliferative control. As indicated in **Figure 1A**, the PA28γ-dependent degradation of several CDK inhibitors (CDKIs) has been observed [61]. Surprisingly, expression of thermo-labile E1 enzymes in ts20TG or tsBN75 cells did not impair degradation of p21 [62]. Furthermore, contribution of SCF E3 ligases in p21 degradation could be excluded, since ts41 cells harboring a temperature sensitive mutation in the NEDD8-activating enzyme did not reveal an increased p21 half-life at the restrictive temperature. However, p21[Cip1] half-life, and similarly that of lysine-less p16[INK4A] or p19[Arf], was extended in embryonic fibroblasts from PA28γ knockout
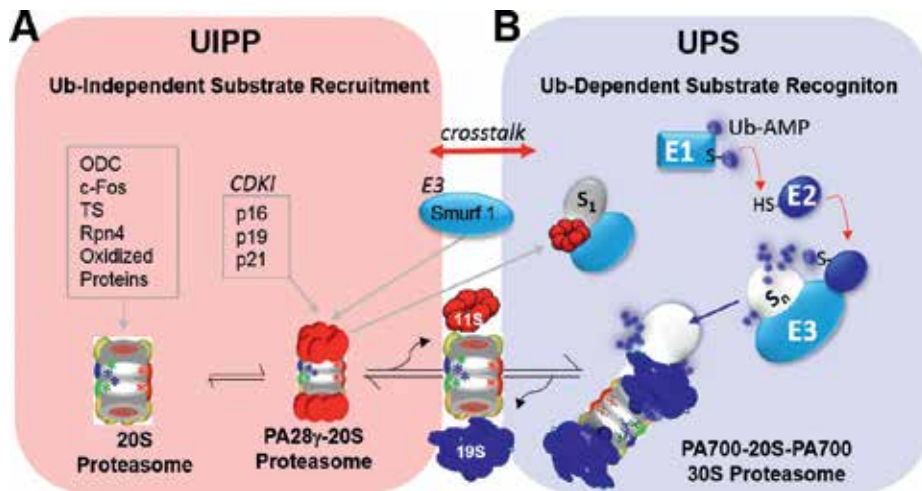
Figure 1. Mechanistic diversity of PA28γ-mediated protein turnover. (A) Typical protease complexes and substrates of UIPP. (B) Core components of UPS. Note, that UIPP and UPS are connected by cross talk: 11S and 19S regulators compete for binding to the 20S core protease. Increased assembly of PA28γ complexes is supposed to reduce the relative amount of 30S proteasomes. In cells overexpressing PA28γ, certain proteasomal substrates of PA28γ-20S proteasomes may be recognized by increased turnover, while others may accumulate due to UPS inhibition. The second mode of cross talk takes place, if UIPP degrades E3 enzymes or components required for their activation. As the third mode, PA28γ might increase PPI between E3s (Mdm2) and their substrates (S1, p53), thereby increasing UPS-dependent substrate turnover.

mice [61]. Noteworthy, aforementioned CDKIs had measurable slow rates of proteasome-dependent turnover after depletion of PA28γ, indicating that PA28γ-20S proteasome UIPP is more efficient than the PA28γ-independent pathway. Furthermore, inhibition of amino-terminal ubiquitination via acetylation or mutagenesis of internal lysine residues did not impair degradation of p21 [62]. Degradation of the three mentioned CDKIs could be performed by 20S proteasomes, but not by 30S proteasomes in vitro [61].

PA28γ-mediated effects can be assigned mechanistically to distinct categories (**Figure 1**). Firstly, central to its role in UIPP, PA28γ recruits substrates for degradation by the 20S proteasome. CDKIs revealing features of intrinsically unstructured proteins (IUP) are prototypic for this mode [61].

Secondly, a cross talk between UIPP and UPS is established, since 11S and 19S regulators compete for binding to 20S proteasomes. Interestingly, overexpression of PA28γ correlates with the increase of some UPS substrates such as c-Myc [63], ERα [58], BclX$_L$, or MART-1 [46]. Such stabilization may indicate inhibitory effects on specific E3 enzymes, UIPP degradation of auxiliary UPS factors, or reduction of the active 30S proteasome pool. Perhaps, putative 30S substrates may escape UPS degradation due to activation of DUBs. Thirdly, UPS-mediated degradation might be affected either by degradation of specific E3 enzymes (Smurf1; [64, 65]) or by enhancing the interaction of specific E3s with their substrates, as has recently been shown for Mdm2:p53:PA28γ [66].

Here, PA28γ promotes the interaction of p53 and Mdm2 (**Figure 1B**), thereby reducing total p53 levels in UV-C-radiated cells via the ubiquitin-dependent proteasomal degradation pathway.

Silencing of the PSME3 gene, as well as application of Nutlin-3, an inhibitor of Mdm2:p53 interaction, resulted in exaggerated stabilization of p53 in HCT116 or A549 cells, respectively [66].

In general, proteasomal contribution to degradation of regulatory proteins has been confirmed by using proteasome inhibitors in an adequate concentration range, thereby stabilizing either the protein of interest (POI) or its ubiquitin conjugates. Ubiquitin conjugates of specific POIs have been demonstrated after transient expression of HA-tagged ubiquitin, HA-pulldown techniques, and consecutive immunoblot analysis. Cellular systems with thermosensitive E1 enzymes were used to demonstrate dispensability of ATP-dependent ubiquitination for some proteasomal substrates. Depletion of subunits crucial for 19S assembly by siRNA silencing and in vitro degradation assays based on purified proteasome complexes either with or without regulators are alternative approaches to demonstrate UIPP or UPS involvement [1].

3.1. PA28γ in DNA damage response

DNA double-strand breaks (DSBs) may lead to chromosomal rearrangements, dysfunctional gene expression, or even aneuploidy, if not properly being targeted by the DNA damage response (DDR). The DDR is a tightly regulated nuclear process, organized majorly by post-translational modification (PTM) signals, implemented by certain stress protein kinases. It has been presumed that DSB mobility and nuclear chromatin dynamics may serve to support DNA repair in nuclear sub-compartments [67]. Major DNA repair pathways are carried out within a highly organized three-dimensional nuclear environment. Homologous recombination (HR) occurs throughout the cell cycle, while nonhomologous end joining (NHEJ) is restricted to S and G2 phases.

Recently, ATM- and PA28γ-dependent recruitment of 20S proteasomes to sites of DSB has been demonstrated by live cell imaging [42]. PA28γ depletion enhanced the focal retention of proteins of the DNA repair machinery (MDC1, 53BP1, RNF8, or BRCA1) at DNA damage sites, whereas early accumulation of focal proteins and initial formation of modified histone γH2AX were not affected. PA28γ silencing moderately reduced the NHEJ pathway, whereas the HR pathway was markedly enhanced. PA28γ seems to assemble proteasomes at the sites of DSB during early stages of the DNA repair pathway [42].

3.2. PA28γ in nuclear dynamics

While varying levels of chromatin compaction result in euchromatin and heterochromatin, nuclear bodies structure interchromatin as well [68]. PA28γ and 20S proteasomes have been found to be physically associated with nuclear structures such as nuclear speckles [40], Cajal bodies (CBs) [41], and PML bodies [69, 70] or have been associated with nuclear survival functions and chromosomal stability [71].

Nuclear speckles as subnuclear interchromatin domains are enriched in components of the pre-mRNA splicing machinery. 20S proteasomes and PA28γ co-localize in such NS structures [40]. PSME3 silencing affects NS organization and recruitment of splicing factors of the SR family to transcription sites. Proteasome inhibitors promote the accumulation of SC35 in NS.

Contributions of UIPP and/or UPS to NS protein dynamics seem to be crucial for nuclear speckle function.

CBs are sites of assembly of small nuclear ribonucleoproteins and small noncoding RNA traffic [72]. They are specific nuclear targets of the cellular stress response [41]. It has been shown that UV-C irradiation induces a stable association of PA28γ with coilin, the intrinsically disordered marker protein of CBs. The accumulation of PA28γ correlates with the disruption of CBs. Apart from its canonical targeting via Mdm2, the unstructured nature of coilin and its association with PA28γ might be the first evidence for coilin targeting via UIPP.

3.3. PA28γ in infection and inflammation

PA28γ and NF-κB signaling constitute activation loops affecting inflammatory processes [73] and bacterial infection [74]. Since inflammatory states are preceding neoplastic transformation, the molecular link between PA28γ and NF-κB biology might be of relevance in tumor biology. The PSME3 gene is under control of the transcription factor NF-κB. Since inhibitory proteins of NF-κB signaling are substrates of a PA28γ-mediated UIPP, NF-κB-mediated transcription depends on PSME3 expression and vice versa. This positive feedback regulation between PSME3 gene regulation and NF-κB-directed transcriptional regulation has been observed in two different models. First, IκBε degradation prevents the removal of NF-κB from sites of transcription, promoting constitutive activation of inflammatory pathways in the gastrointestinal mucosa. A DSS colitis model demonstrated the requirement of PA28γ for the pathological process. PSME3 knockout resulted in impairment of the DSS colitis pathology [74]. The second target of PA28γ in NF-κB signaling is KLF4, a negative regulator of NF-κB. Targeting of KFL4 by the PA28γ-proteasome releases the negative modulatory effect on NF-κB, resulting in a higher PSME3 expression and further activation of the inflammatory response to counterstrike bacterial infection [73].

Accessing sera of autoimmune and cancer patients, we observed an increased level of extracellularly localized PA28γ in patients suffering from a diverse spectrum of autoimmune diseases. In patients with rheumatoid arthritis, the level correlated with the disease state and responded to treatment with abatacept [49]. Since PA28γ has been associated with sepsis [75] or wound healing [76], development of clinical applications beyond autoimmune or cancer diagnosis is an emerging future perspective.

3.4. Transcriptional regulation of metabolism

Transcription factor c-Myc, a highly unstable protein [63, 77, 78], acts as a heterodimer with Max, recruiting coactivators through CACGTG-binding motifs to promotors of target genes involved in the regulation of cell growth, proliferation, metabolism, and apoptosis. Interestingly, c-Myc appears to be targeted by diverse PTMs and proteolytic mechanisms. UPS-dependent degradation of c-Myc depends on several E3 enzymes, F-Box proteins, and antagonizing pathways [79]. Using gene set enrichment analysis, PA28γ was shown to reprogram energy metabolism via the c-Myc-glycolysis axis, affecting glucose utilization and lactate production in cancer cells. PSME3 gene silencing reduced c-MYC levels, whereas the increased levels of

PA28γ in cancer cells increased Myc. High levels of PA28γ protein in the cytosol and nucleus repress UPS-mediated c-Myc decay and reprogram pancreatic tissue and pancreatic cancer cell lines metabolically [80]. These results were surprising, since overexpression of PA28γ in Hela cells promoted degradation of c-Myc, while depletion of PA28γ markedly increased the protein stability of c-Myc [51]. These contradictory observations indicate contextual variability of c-Myc turnover in pancreatic tumors versus other models and underline the requirement for studies focusing on cross regulation of UIPP and UPS.

3.5. PA28γ: Impact on autophagy and metabolism

Highly selective proteolytic systems such as UPS or UIPP, responsible for the selective and regulated degradation of proteins, are supplemented by autophagosomes and ub-based substrate labeling for cargo selection. Ubiquitin-like proteins and a corresponding conjugation system are initially involved in phagophore formation, a process, which integrates phosphatidylethanolamine-linked LC3-II into both leaflets of the autophagosome. Cargo receptors for ubiquitinated proteins such as p62 are recruited to autophagosomes via LIR domains (LC3-interacting region (LIR)). Finally, degradation of bulky aggregates or organelles can be conducted by lysosomal proteases during basal autophagy. Under environmental stress conditions, as an adaptive survival strategy, autophagy can be gradually upregulated, avoiding excessive activity of autophagy which may lead to cell death. The regulation of this sophisticated machinery for bulk degradation has recently been reviewed [81]. Interestingly, since Bcl-2 family proteins are closely linked to cytoprotective responses, regulation of metabolism, and apoptosis, the involvement into regulation of autophagy is not surprising. Generally, anti-apoptotic members such as BclX$_L$ can inhibit autophagy, whereas pro-apoptotic BH3-only proteins may induce autophagy. The principal behind anti-apoptotic or anti-autophagic roles of BclX$_L$ may be considered simply the same, while complexity in cross talk between autophagy and apoptosis majorly results from BclX$_L$ phosphorylation and subcellular localization. BclX$_L$ has been reported to be localized in the cytosol, the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane, establishing BclX$_L$ pools of different functionalities. In anti-apoptotic signaling, BclX$_L$ antagonizes pro-apoptotic proteins via physically interacting with Bax, cytochrome c, or cytosolic p53, preventing MOMP [82, 83]. Binding of Beclin 1 to BclX$_L$ prevents assembly and activation of class III phosphatidylinositol 3-kinase (PI3K) complex, which is involved in autophagosome function.

Similarly, multifaceted as pro-survival factor BclX$_L$ [82], anti-apoptotic PA28γ protein reveals a plethora of PPIs and functional versatility. Targeting of deacetylase SirT1 for PA28γ-20S proteasome-mediated UIPP degradation was shown to regulate liver autophagy [84]. Deficiency of PA28γ as well as energy starvation dissociates the REGγ-SirT1 interaction and releases SirT1 to deacetylate components of the autophagy machinery, thereby stimulating autophagy.

Analyzing metabolic parameter of PA28γ knockout mice, a reduction of ATP consumption, possibly due to an inhibition of rDNA transcription, has been demonstrated. Under starvation, nucleolar deacetylase SirT7 was negatively regulated by PA28γ. Consistently, depletion of PA28γ induced increased Sirt7 levels, as well as increased ATP consumption. These observations

might be of relevance for cancer therapy, since PA28γ depletion sensitizes tumors to treatment with hexokinase inhibitor 2-desoxy-glucose [85].

3.6. Cross talk of UIPP and UPS in the regulation of apoptosis

3.6.1. Stoichiometry of 20S proteasomes and regulators in cellular systems

The stoichiometry of the key regulators of UPS and UIPP, namely, 19S and 11S regulators, is of particular interest to further understand how cross talk of these two proteolytic systems is affected by PA28γ. Since each biochemical process is restricted kinetically by its rate limiting step, the assembly of proteasomal complexes should be a matter of serious debate. Only few studies so far covered this question due to methodological limitations. Therefore, a recent proteomic approach is remarkable, elaborating the stoichiometry of 20S proteasome-associated regulators in detail [86, 87]. Using formaldehyde cross-linking in combinations with 20S-complex directed affinity purification on MCP21-coupled sepharose, and applying mass spectrometric quantification, proteomic analysis compared the stoichiometry of 20S proteasome complexes in nine different cell lines. Interestingly, 19S and 20S particles reached 1:1 ratio, but PA28γ-associated proteasomes occurred as minor species, occupying about 1–0.2% of proteasomes [86]. All investigated cellular systems had in common that about 20–40% of 20S proteasomes persisted in a regulator-unbound state, establishing a pool of proteasomes directly available for UIPP tasks or for dynamic behavior in the case of expressional alterations in the activator population [86].

As central pathways of protein degradation, UPS and UIPP participate in regulating the delicate balance of pro- and anti-apoptotic proteins and cell cycle regulators [88, 89]. The contribution of proteasomal degradation in controlling crucial steps of the mitochondrial pathway of apoptosis has been reviewed comprehensively [89]. Here, we are focusing on the PA28γ-mediated mechanisms, affecting the balance between pro- and anti-apoptotic regulatory proteins (**Figure 2**).

3.6.2. Discovery of anti-apoptotic properties of PA28γ

Initial studies on PA28γ-deficient mice suggested a role for PA28γ as a regulator of cell proliferation and body growth [90]. Lack of PA28γ did not affect expression of other PSME family members such as PA28α or PA28β and resulted in smaller body size. Entry into S phase was impeded and number of G1 cells increased. MEFs depleted in PA28γ revealed increased spontaneous apoptosis during logarithmic growth.

Recently, we demonstrated a correlation between cellular PA28γ levels and the sensitivity of cells toward apoptosis in different cellular contexts, thereby confirming a role of proteasome activator PA28γ as an anti-apoptotic regulator [46]. We investigated the anti-apoptotic role of PA28γ upon UV-C stimulation in B8 mouse fibroblasts stably overexpressing the PA28γ-encoding PSME3 gene and upon butyrate-induced apoptosis in human HT29 adenocarcinoma cells with silenced PSME3 genes. Interestingly, our results demonstrate that PA28γ has a strong influence on different apoptotic hallmarks, especially the levels of transcriptionally active phosphorylated p53, BclX$_L$, and active effector caspases (**Figure 2**) [46].

3.6.3. Connecting the p53 activity status and PA28γ biology

The biology of p53, a key regulator of DDR and apoptosis, appeared recently being intimately connected to PA28γ. Our data on UV-C-induced apoptosis in murine fibroblasts overexpressing PA28γ revealed an increase of phosphorylated nuclear p53, paralleled by cytosolic disappearance of p53. Kinetic experiments of apoptosis induction showed that protein levels of pro-apoptotic Bcl-2 family member Bax, cell cycle inhibitor p21, and ubiquitin E3 ligase MDM2 were transcriptionally upregulated in a time-dependent manner, indicating an increased level of transcriptionally active p53 in a pro-apoptotic context.

Furthermore, it was observed in endometrial cancer that mutant p53[R248Q] promoted the upregulation of the PSME3 gene [91]. That the PSME3 gene locus is under inhibitory transcriptional control has been reported too. Wild-type p53 inhibits the PA28γ-20S UIPP pathway by repressing PSME3 gene transcription, whereas mutant p53 was unable to repress PSME3 transcription [92].

Among other cellular survival functions, the p53 tumor suppressor protein particularly regulates key decision points of DNA repair and apoptosis via affecting transcriptional regulation of pivotal regulators of these two processes. Under normal conditions, the p53 protein is constitutively degraded via Mdm2-mediated ubiquitination and the 30S proteasome (**Figure 2D**). Under non-apoptotic conditions, PA28γ facilitates cytosolic redistribution of p53 by enhancing its nuclear export via a mechanism involving mono-ubiquitination [53]. Cytosolic p53 can activate MOMP via Bax, a mechanism prevented or balanced by the p53:BclX$_L$ and BAX: BclX$_L$ interactions [82]. Similarly, a release of cytochrome c into the cytosol, triggering apoptosome formation (**Figure 2A**), can be antagonized by Cyt c:BclX$_L$ [83] or p27:Cyt c interaction (p27, small heat shock protein), while other heat shock proteins counteract via interaction with Apaf-1 (**Figure 2A**). One key regulator in this picture is BclXL, which responds transcriptionally to p53 activation and is posttranscriptionally downregulated by PARK2, the specific E3 ubiquitin ligase preparing BclX$_L$ for UPS-mediated degradation (**Figure 2B**). Another anti-apoptotic regulator of MOMP, Mcl-1, is similarly targeted for degradation by Mule E3 ubiquitin protein ligase (**Figure 2B**) [82, 89].

DNA damage-induced ATM protein kinase (**Figure 2D**) activates p53 by phosphorylating p53 and Mdm2, thereby regulating Mdm2 oligomerization and processivity [93]. As a result of site-specific phosphorylation (human p53 [Ser15P] or mouse p53[Ser18P]), p53 becomes stabilized and translocates into the nucleus. Phosphorylated wild-type p53 tetramers are capable of promotor and coactivator/corepressor binding [94].

In healthy cells, p53[wt] proteins cooperate with Smad3 in repressing the PMSE3 promotor [92]. Contrarily, tumor cells reveal high levels of p53[mut] activating the PMSE3 promotor via derepression and/or activation with unknown coactivators. Consistently, elevated PA28γ levels have been detected in several types of cancer and tumor cell lines [92]. These PA28γ-overexpressing cells, hence, reveal higher resistance to apoptotic stimuli and increased proliferation.

In cancer cells, mutant p53 (p53[mut]) variants reveal alternative promotor selectivity. This might explain how irreparable DNA damage in healthy cells results in transcriptional amplification
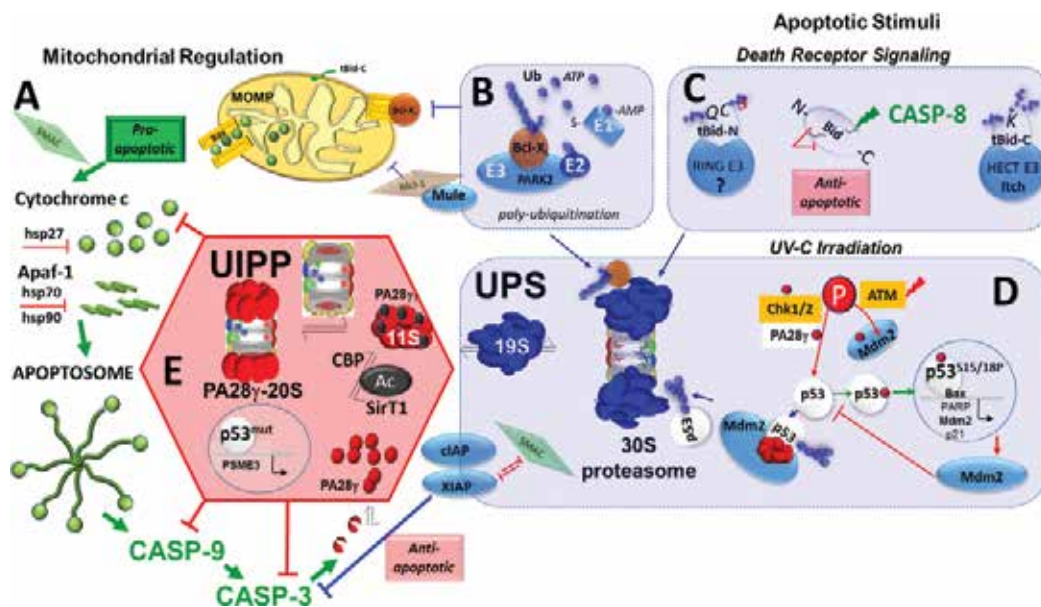
Figure 2. Impact of PA28γ on UIPP- and UPS-mediated regulation of apoptosis. (A) Mitochondrial regulation of apopto-sis via MOMP, apoptosome assembly, and caspase activation. (B) Anti-apoptotic role of BclX$_L$ and its control via the UPS. (C) Death receptor signaling and extrinsic activation of apoptosis via caspase-8 (CASP-8) depend on the intriguing UPS-mediated regulation of the bid protein (reviewed by [89]). CASP-8 cleavage of bid generates amino- and carboxy-terminal peptide fragments (N-tBid; t-bid-C) that act antagonistically. Anti-apoptotic N-tBid still bound to pro-apoptotic tBid-C prevents apoptosis. Noncanonical ubiquitination at Gln and Cys residues initiates UPS-mediated degradation of N-bid. N-bid degradation is required for activation of pro-apoptotic tBid-C. Inactivation of t-bid-C is realized by itch E3 ubiquitin ligase, initiating its UPS-mediated degradation. This prevents mitochondrial outer membrane pore formation and activa-tion of apoptosis. (D) Induction of stress kinases ATM and Chk2 by UV-C irradiation induces phosphorylation of p53, Mdm2, and PA28γ, thereby disconnecting p53 from its constitutive restriction through E3 ubiquitin ligase Mdm2. Phosphorylated nuclear p53 activates transcription of pro-apoptotic Bax, and cytosolic p53 can activate Bax-mediated pore formation directly. It has been observed that PA28γ reduces p53 levels by enhancing Mdm2:p53 interaction [66]. (E) Our model proposes a central role of anti-apoptotic PA28γ-20S UIPP in cancer cells [46]. High levels of PA28γ might be based on altered promotor selectivity of mutant p53, which has been shown to increase PSME3 transcription, whereas wild-type p53 represses PSME3 gene expression (D) [91, 92]. High PA28γ levels and acetylation are favoring heptamer assembly and proteasome activation. UIPP, either based on 20S proteasomes alone or in association with PA28γ, may reduce the level of released cytochrome c or activated caspases (CASP-9; CASP-3). If PA28γ levels are low, restriction of caspases by UIPP is released, and monomeric PA28γ is targeted by effector caspases (CASP-3). Of note, experimental evidences support the proteasomal degradation of effector caspases [46, 95], but the precise role of PA28γ in this process remains to be investigated in detail.

of apoptosis due to increasing expression of pro-apoptotic genes, while in cancer cells an anti-apoptotic scenario prevails.

3.6.4. Feedback regulation: PA28γ inhibits effector caspase activity

Overexpression of the PSME3 gene was accompanied by an increased resistance to apoptosis induction. Even with elevated levels of BclX$_L$ and a partially impaired cytochrome c release in PA28γ-overexpressing cells, we observed execution of the caspase-9/caspase-3 activation cascade upon UV-C stimulation [46]. Surprisingly, PA28γ overexpression correlated with significantly

decreased active effector caspase levels. Consistently and vice versa, PMSE3 miRNA-mediated PA28γ downregulation was accompanied by increased sensitivity to butyrate-triggered apoptosis of HT-29 cells and increased levels of active caspase-3/caspase-7 [46]. Reduced caspase activities were not due to transcriptional but posttranslational regulation.

The anti-apoptotic impact of high PA28γ levels could not solely be explained by enhanced PA28γ-mediated degradation of pro-apoptotic p53 as suggested by others [66]. Our current findings support a model (**Figure 2A** and **E**), where high PA28γ levels inhibit effector caspase-3/caspase-7, while at low PA28γ levels, caspase-3/caspase-7 activity and PA28γ turnover are increased. As a reasonable explanation, the high level of PA28γ favors heptamer stability, whereas at low concentrations, monomeric PA28γ may be more susceptible to cleavage by activated effector caspases [46, 47]. Furthermore, we found that proteasome inhibition stabilized active caspase-3 levels, indicating that the PA28γ-dependent degradation of caspase-3/caspase-7 is indeed proteasome-dependent [46]. Since the RING domain of IAP proteins ubiquitinates caspase-3/caspase-7 [95], the canonical UPS is certainly involved in regulating effector caspase activity. If PA28γ conducts restriction of caspase-3/caspase-7 activity through enhancement of physical interaction between IAPs and caspases, or directly via the PA28γ-20S proteasome UIPP route, has to be clarified in the future.

4. Concluding remarks

In light of the fact that the apoptotic potential of the cell is finally restricted by caspase levels and their execution efficiencies [96], the importance of check and balance for controlling caspase activity appears to be obvious. Recently, the impact of posttranslational modification on activity and stability of caspase-3 has been reviewed [97]. Apart from inhibitory and stimulatory phosphorylation, ubiquitination plays an important yet not a fully understood role. However, cIAP1-dependent ubiquitination of a processing intermediate of caspase-3 was followed by proteasome-dependent degradation of caspase-3. Interestingly, proteasome inhibitors stabilizing majorly active caspase-3 among other effector caspases enhanced apoptosis [97].

The integration of the recent knowledge on proteasomal contribution to regulation of apoptosis via UIPP and UPS might lead to a refined concept in system biology of apoptosis [98]. Despite of the remaining questions, experimental evidences indicate that tumor survival and resistance to therapeutic approaches may crucially relate to a plethora of new roles of PA28γ in the regulation of apoptosis, autophagy, inflammation, and metabolic adaptation.

Acknowledgements

Author details

Ralf Stohwasser

Address all correspondence to: ralf.stohwasser@b-tu.de

Brandenburg Technical University Cottbus-Senftenberg, Senftenberg, Germany

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Signaling Pathways Targeted by Protozoan Parasites to Inhibit Apoptosis

Laila Gutiérrez-Kobeh, Jorge Rodríguez González,
Rosalino Vázquez-López and
Arturo Wilkins-Rodríguez

Additional information is available at the end of the chapter

Abstract

Apoptosis is a biological process carried out during maturation, remodeling, growth, and developmental processes in tissues, and also represents an important defense mechanism of cells against intracellular microorganisms. In counterpart, diverse intracellular pathogens have developed a wide array of strategies to evade apoptosis and persist inside cells. Apoptotic cell death can be triggered through different intracellular signaling pathways that lead to morphological changes and eventually cell death. Among these pathways, MAPK and PI3K play a central role. The precise control of the signaling pathways that lead to apoptosis is crucial for the maintenance of tissue homeostasis. Paradoxically, these same pathways are utilized during infection by distinct intracellular microorganisms in order to evade recognition by the immune system, inhibit apoptosis, and therefore survive, reproduce, and develop inside cells.

Keywords: apoptosis, inhibition, protozoan parasites, signaling pathways

1. Introduction

The word apoptosis has its etymological origin in the Greek *apó*, which means "from" and *ptōsis* which means "falling off." The merging of these two words is an allusion to the natural events of shedding cells and tissues, as well as the falling of old leaves during autumn. Apoptosis describes the process in which unwanted, damaged, or old cells are eliminated in multicellular organisms [1], which is necessary in all body tissues and happens naturally during

embryogenesis, metamorphosis, and constant cellular changes, being of upmost importance for the maintenance of homeostasis in all tissues [2]. The term apoptosis was coined since 1972 by Kerr, to define a type of programmed cell death with morphological and molecular characteristics different from other types of cell death. These characteristics include retraction of pseudopods with the consequent reduction of cellular volume and rounding of the cell, nuclear volume reduction (pyknosis) and fragmentation (karyorrhexis), structural modification of organelles followed by the formation of vesicles due to blebbing of the plasma membrane [3, 4]. Apart from being a fundamental process of cells for the maintenance of homesostasis, apoptotic cell death represents an important defense mechanism against intracellular pathogens. Against it, a wide spectrum of microorganisms has developed diverse strategies to inhibit apoptosis of their host cells. These strategies involve different signaling pathways that are hijacked by pathogens to achieve their goal of inhibiting apoptosis and persist inside cells. The purpose of this chapter is to better understand the signaling pathways that are targeted by protozoan parasites in order to evade the defense mechanism of apoptosis.

2. Generalities of apoptosis

2.1. Initiation of apoptosis

The activation of apoptosis requires the assembly of an intricate web of intracellular signaling pathways that occurs in three phases: initiation or activation, execution, and cellular demolition that are triggered in three different ways: the extrinsic pathway, the intrinsic pathway (subdivided in mitochondrial-induced apoptosis and endoplasmic reticulum stress-induced apoptosis) and the caspase-independent pathway [5–9].

2.1.1. The extrinsic pathway

This pathway is activated through extracellular stress signals that are detected and amplified by transmembrane receptors called death receptors [10–12]. Some of these receptors include the Tumor Necrosis Factor receptor (TNFR), Fas receptor (CD95), DR3/WSL, and Apo-2L (TRAIL-R1/DR4, TRAIL-R2/DR) [13, 14], which are characterized for the presence of intracellular domains called death domains (DD), which include the TNFR or TRADD and Fas or FADD death domains [15]. Once receptors become engaged with their respective ligands, activating proteins such as RIPK1, FADD, c-FLIP, c-IPAs, and ubiquitin ligase E3 are recruited [16–21], and in consequence, a supramolecular complex is formed by the activating protein-receptor domain that is recognized as a Death-Inducing Signaling Complex (DISC), which activates procaspase 8, the precursor of caspase 8 [16, 18–22]. In some cases, the extrinsic pathway can be triggered without a ligand as is the case of DCC and UNC5B receptors where, in the absence of a ligand, DCC interacts with cytoplasmic adapting protein DRAL to assemble an activation platform for caspase 9 [23]. In a similar manner, the UNC5B receptor, in the absence of netrins, recruits a molecular complex composed of PP2A and Death Associated Protein Kinase 1 (DAPK1) [24]. In both cases, caspase 8 is activated to initiate cell death via apoptosis.

2.1.2. The intrinsic pathway

The mitochondrial intrinsic pathway can be initiated by different intracellular stimuli such as irreversible genotoxic damage, increase in the cytoplasmic calcium (Ca^+) concentration, oxidative stress, among others [15]. In this pathway, a family of proteins called Bcl-2, characterized for having from 1 to 4 conserved domains that share homology with Bcl-2 or BH [6], has a leading role. This family is composed of proapoptotic proteins that, according to the BH domains that possess, are divided into Bax and "BH3 only" subfamilies. The members of the Bax subfamily are Bak, Bax, Bok, and Mtd and possess three BH domains (BH1-BH3), while the "BH3 only" subfamily, as denoted by its name, possesses a single BH3 domain and is composed of Bid, Bad, Bim, Bik, Blk, Hrk, NOXA or PUMA. On the other hand, the antiapoptotic proteins family present four BH domains (BH1-BH4) and is composed of Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1 [6]. The BH1 and BH2 domains are structurally similar to the diphtheric toxin [25, 26]. The antiapoptotic proteins Bcl-2 and Bcl-xL are located in the outer mitochondrial membrane and prevent the release of cytochrome c, while the proapoptotic proteins Bad, Bid, Bax, and Bim are located in the cytosol and under certain stimuli are translocated to the mitochondria, where they induce the release of cytochrome c [25, 26]. Additionally, caspase 8 may take part in the intrinsic pathway through Bid proteolysis, turning it into tBid, which also translocates to the mitochondria and activates Bcl-2, Bax, and Bak [27]. Once Bax and Bak have been translocated to the mitochondrial membrane, a molecular complex referred to as PTPC is activated and induces the Mitochondrial Transition Permeability (MTP) phenomenon [28, 29]. These events culminate in the permeabilization of the outer mitochondrial membrane or MOMP, which is the rate-limiting step in apoptosis that conducts to an energetic and metabolic damage and the cell faces irreversible apoptotic cell death. The release of cytochrome c from the mitochondrion permits its association with the Apoptosis Activation Factor (Apaf-1) thus forming a structure to which procaspase 9 is incorporated, originating a molecular complex referred to as the apoptosome. As procaspase 9 is activated, it recruits executor caspases 3 and 7, which causes a proteolytic effect inducing cell death [27]. As mentioned earlier, the intrinsic pathway can also be activated via endoplasmic reticulum stress whose main stimulus is the misfolding of proteins and their subsequent accumulation in the endoplasmic reticulum (ER). Once misfolded proteins reach a critical concentration, they activate ER membrane sensors [30].

The induction of apoptosis conducts hopelessly to the activation of caspases; nevertheless, the damage to the mitochondria can, in some cases, provoke the release of some molecules with proapoptotic capacities such as HTRA2, AIF and ENDOG that have the ability to induce apoptosis without the intervention of caspases. HTRA2 has the ability to attack proteolytically the cytoskeleton, while AIF and ENDOG can enzymatically attack DNA [31].

2.2. Caspases

Caspases (**C**ysteine-dependent, **ASP**artate-specific peptid**ASE**) owe their name to the fact that their proteolytic functions lie specifically in an aspartate residue and require the presence of cysteine to perform their catalytic activity [6]. There are many types of caspases and are classified according to their function in initiation caspases: 2, 8, 9, and 10; executor caspases: 3, 6,

and 7; and inflammatory caspases: 1, 4, and 5. In addition, there are other caspases that perform diverse functions such as caspase 11, which regulates cytokines during septic shock, caspase 12 that is associated with endoplasmic reticulum stress apoptosis, and caspase 14, which has only been isolated in embryonic tissue, specifically in keratinocytes. Caspases are found in cells in an inactive state called zymogens or procaspases that possess three distinct regions: a prodomain located in the N-terminal end, a minor subunit close to the C-terminal end and, in between, the major subunit. Procaspases are activated through autoactivation or activation by another caspase or molecule that cause the excision in two sites of the aspartate residues, the first one between the prodomain region and the major subunit, and the second between the major and minor subunits [32]. The activation of caspases starts with an initiator caspase that requires the formation of a multimeric adaptor protein complex called apoptosome whose formation is mediated by Apaf-1, an inactive monomer in nonapoptotic cells [31]. MOMP-mediated release of cytochrome c triggers the formation of the apoptosome through the binding of cytochrome c to Apaf-1 on the WDR domain, following by the conversion of ADP into dATP/ATP in the NOD domain [33–35]. Finally, procaspase 9 binds to Apaf-1 through a homotypical interaction with the CARD domains [36]. The apoptosome catalyzes the autoproteolytic action of procaspase 9, and its active form, caspase 9, remains active and bound to Apaf-1 as a holoenzyme [36].

2.3. Cellular demolition

Once apoptosis is triggered through one of the different pathways just explained, the activation of caspase 9 unchains a cascade of executioner caspases [6, 15], whose proteolytic action is directed to multiple substrates that finally culminate in the demolition of the cell. One central substrate targeted by caspases is ROCK1, an actin cytoskeleton activity regulator that upon activation loses its C-terminal end, subsequent phosphorylation, and thus activation of the myosin for is subsequently phosphorylated, and thus activates the myosin light chain, which generates actin contraction that in turn triggers several phenomena such as phosphatidylserine translocation, cellular rounding and retraction, as well as vesicle formation or blebbing and loss of intercellular unions due to the proteolytical attack of desmosomes or other forms of cell to cell junctions. It also affects nuclear membrane integrity and provokes further fragmentation of DNA and degradation of proteins associated with transcription and translation [6, 37–49]. Other targets attacked by caspases are, for example, the caspase-activated DNase (CAD), whose activation culminates in DNA degradation at internucleosomal sites [49] or Golgi reassembly and stacking proteins (GRASP)that participate in Golgi apparatus conformation, cistern formation and connections leading to Golgi fragmentation and disintegration [6, 50]. Continuing with the demolition events, the mitochondrial proteins Bax and Bak are activated due to BH3 action, which in turn generate pores in the mitochondrial membranes and release of their contents. Also, the p75 subunit of the electron transport chain complex 1 is proteolytically degraded [6, 50]. One of the final acts of apoptosis is the release of chemotactic cytokines and other molecules, as well as the formation of union sites for phagocytic cells indispensable for the elimination of cellular remains by phagocytes for these cells [6, 51].

3. Signal transduction pathways in apoptosis

3.1. MAPK family

For apoptosis to be carried out an orchestrated array of signal transduction pathways needs to be put into action among which mitogen-activated protein kinases (MAPK) play a leading role.

These mitogen-activated protein kinases, as denoted by their name, are activated not only by mitogens but also by other physical and chemical stimuli, such as growth factors, UV radiation, genotoxic agents, oxidative stress, inflammatory signals, and cytokines. Once activated, MAPK go through three secuencial phosphorylation steps [52], carried out by three groups of enzymes: (1) MAPK kinase kinase (MAPKKK or MAP3K), for example ASK1; MAPK kinase (MAPKK), for example MEK 1 through 7; MAPK such as ERK 1/2, JNK, and p38. MAPKs belong to the serine-/threonine-type kinases [53, 54] and possess tyrosine (Tyr) and threonine (Thr) conserved double phosphorylation domains [52]. They are further divided in three subfamilies according to the amino acid present in both phosphorylation sites (Thr-XXX-Tyr) [53–55]:

1. The p38-MAPK subfamily features glycine between the two phosphorylation sites (Thr-Gly-Tyr) and is activated through stress signals, growth, and differentiation factors. This subfamily is composed of the p38-MAPKα, p38-MAPKβ, p38-MAPKγ, and p38-MAPKδ isoforms that share a 12-amino acid activation loop and differ in affinity for the activating protein, tissue expression, and downstream effect. The p38α isoform, commonly referred to as p38, as well as the p38β isoform are ubiquitous being present in almost every tissue, while p38γ and p38δ isoforms have a more restricted localization. When p38 is activated, it initiates the three rounds of phosphorylation that culminate in the phosphorylation of p38 specifically at Thr180 and Tyr182 sites. This phosphorylation process produces conformational changes that lead to the enzyme binding with ATP and the acceptor substrate of the phosphate [56]. This subfamily participates in the regulation of certain growth factors, kinases and phosphatases, as well as in the regulation of ATF-2, MEF2, MAPKAPK, CDC25 or MSK1/2 and their activation triggers cellular proliferation, differentiation, apoptosis, among others [57, 58].

2. The JNK subfamily features proline between the two phosphorylation sites (Thr-Pro-Tyr) and is composed by the JNK1, JNK2, and JNK3 isoforms. These proteins are also known as stress-associated MAPKs or SAPKS (stress-activated protein kinases) and participate in cellular growth, differentiation, and apoptosis [59, 60] as a response to diverse stress signals, such as UV or gamma radiation, protein synthesis inhibitors (anisomycin), hyperosmolarity, toxins, ischemic damage, thermal shock, antineoplastic drugs, peroxides, and inflammatory cytokines, among others [59]. Stress signals initiate the three cycles of phosphorylation with the activation of MAP3K, ASK1 and ASK2, among others, which in turn activate MEK4 and MEK7 through phosphorylation of two specific serine and threonine residues. Finally, MEK4 and MEK7, also known as MKK4 (SEK1/JNKK1) or MKK7 (SEK2/JNKK2) phosphorylate JNK in threonine-proline-tyrosine (Thr-Pro-Tyr) specific residues [59, 61, 62]. Interestingly, the biological roles of JNK isoforms are similar [63], although they are physically different and also differ in tissue localization. JNK1 and JNK2 are expressed in all tissues, while JNK3 isoform is found predominantly in nervous tissue, and to a lesser extent in the heart and sperm [64–66]. Although JNK1, JNK2, and JNK3 can all induce apoptosis, there is evidence suggesting that each protein induces apoptosis through a different pathway. It has been demonstrated that all of them associate with p53, a nuclear transcription factor that activates proapoptotic gene expression, such as BAX or PUMA, but interestingly their expression varies with respect to p53. In the case of JNK1, its expression is inversely proportional to p53, contrary to JNK2 expression, which is directly proportional to p53. Both JNK2 and JNK3 can phosphorylate p53, while JNK1 can only modify it post-transcriptionally [67, 68].

3. The ERK subfamily features glutamic acid between the two phosphorylation sites (Thr-Glu-Tyr) and is composed of ERK1, also known as MAPK3, and ERK2, also known as MAPK1 or p42MAPK [62, 69]. These kinases are activated by growth factors, hormones,

and neurotransmitters through binding to different G-protein coupled receptors, tyrosine-kinase receptors, and ion channels. Then, signal transduction continues with an adaptor protein that transmits the signal to a MAP3K of which several have been described for ERK such as Raf-1B-Raf, A-Raf, and TPL2 [62]. Following the described phosphorylation pattern (MAPKKK → MAPKK → MAPK), the stimulus activates MAPKKK (i.e., Raf-1), which in turn phosphorylates MEK1 and MEK2 (both MAPKK) and these finally phosphorylate and activate ERK1 and ERK2 [62].

3.2. MAPK participation in apoptosis

One of the upmost actions of MAPK is the activation of transcription factors, which regulate the expression of genes that lead to crucial molecular events in the cell regarding growth, proliferation, inflammatory cytokine production, and apoptotic cell death [56]. In relation to apoptosis, a key participant is JNK that plays its role through two different mechanisms. The first one is related to nuclear events in which JNK is translocated to the nucleus and activates c-Jun and other transcription factors that promote proapoptotic gene expression, through p53/73 or c-Jun/AP1-dependent mechanisms [70, 71]. The second mechanism relates to JNK activation and translocation to the mitochondria, where it promotes the phosphorylation of protein 14–3-3, a protein that normally inhibits Bax by being bound to it. As protein 14–3-3 is phosphorylated, Bax is released and translocates to the interior of the mitochondria where it oligomerizes and forms pores in the mitochondrial membrane with the subsequent release of cytochrome c and apoptosis induction through the intrinsic pathway. Apart from these two mechanisms, JNK can also phosphorylate "BH3-only" family members, whose antiapoptotic effect inhibits Bcl-2 and Bcl-xL and is also involved in the posttranslational modifications of Bid and Bim, both of which induce Bad and Bax activity [70, 71]. Another MAPK deeply involved in apoptosis is p38, which in many times is simultaneously activated with JNK [72]. p38 exerts its central role in apoptosis through the activation of proapoptotic proteins, mainly BimEL, BAD, and Bax [73–77] and simultaneously induces the inhibition of ERK and Akt antiapoptotic pathways [76, 77]. Also, p38 and JNK participate in TLR signaling pathways. These key participants of the innate immune response function as regulatory sensors of both apoptosis signaling through the induction of MAPK p38 and JNK [78, 79] and survival signals through PI3K and some Bcl-2 family members [80–82].

4. PI3K/Akt signaling pathway and its participation in apoptosis inhibition

As previously mentioned, MAPK p38 and JNK play an important role in apoptosis induction. On the other hand, PI3K activation promotes cellular survival. PI3K is a heterodimer formed by a p85 regulatory subunit and a p110 catalytic subunit responsible for phosphate transfer. The signaling pathway initiated by this kinase is activated by different stimuli, with growth factors standing out among them. Once a ligand binds to the tyrosine specific tyrosine-kinase receptor, an IRS adaptor protein is activated, which in turn activates the regulatory PI3K subunit and generates a conformational change that allows the binding of the catalytic subunit and thus the assembly of the active molecule that catalyzes the conversion of PIP_2 into PIP_3

[83, 84]. PIP_3 interacts with the pleckstrine homology (PH) domain, located in the N-terminal region of the serine/threonine kinase Akt or PKB, with the final result of the kinase being recruited to the plasma membrane [85–87]. Furthermore, PDK1 phosphorylates Akt/PKB producing a conformational change that facilitates a second phosphorylation by the rictor-mTOR1 complex [88]. Finally, the PI3K/Akt pathway leads to diverse effects associated with cellular proliferation and survival [89, 90]. Specifically, it produces the inactivation of many proapoptotic signals, such as BAD, procaspase-9, and FKHR (Forkhead) transcription factors [21, 91]. It also promotes the activation of CREB, NF-κB, and HIF-1α transcription factors, which in turn activate the expression of antiapoptotic genes [92–94].

5. Apoptosis inhibition and infection

Apoptosis constitutes a very important defense mechanism against intracellular microorganisms [95], whom in order to survive inside cells need to inhibit the induction of apoptosis. It has been demonstrated that diverse intracellular pathogens including virus [96], bacteria [97], and protozoan parasites [98] have developed mechanisms to persist within host cells without inducing apoptosis.

5.1. Inhibition of apoptosis by *Leishmania*

Leishmania is an obligate intracellular parasite that infects a variety of cells such as neutrophils, macrophages (Mφ) and dendritic cells (DC). *Leishmania* has developed diverse mechanisms to manipulate host cells in order to evade the immune response and survive inside cells. Some of these strategies are the evasion of the phagosome-lysosome fusion and the inhibition of apoptosis. Studies have demonstrated that monocytes, macrophages, and dendritic cells grown in apoptogenic conditions and infected with different species of *Leishmania* present an inhibition of normal apoptosis. Also, *Leishmania* infection prevents natural apoptosis of neutrophils. The first demonstration of the inhibition of apoptosis by *Leishmania* was performed by Moore and Matlashewski in 1994 who demonstrated that the infection of bone marrow derived-macrophages (BMM) with *Leishmania donovani* promastigotes or the stimulus with LPG inhibited apoptosis induced by the deprivation of M-CSF. Interestingly, the culture supernatant of infected BMM was able to inhibit apoptosis suggesting that the effect could be due to soluble mediators [99]. A later study showed that cellular activation increased the production of TNF-α, TGF-β, IL-6, and GM-CSF, while the secretion of M-CSF and IL-1β diminished [100]. Studies performed later with another species, *Leishmania major,* showed that the infection of macrophages grown in the absence of M-CSF or in the presence of staurosporine inhibited the release of mitochondrial cytochrome c, thus delaying apoptosis. It was observed that the infection of BMM with *L. major* promastigotes inhibited caspase-3 activation owed to a decrease in MOMP and subsequent release of cytochrome c, which was not associated to NF-κB activation since the use of specific inhibitors did not affect the capacity of L. major to inhibit macrophage apoptosis. It was also demonstrated that the infection of BMM obtained from BALB/c o C57BL/6 mice with *L. major* promastigotes preserved the phenomenom of apoptosis inhibition despite the genetic background of the host or type of immune response (Th2 or Th1, respectively) [101]. Also, studies performed with cell lines reported

similar results as in the case of the monocyte cell line U937 infected with *Leishmania infantum* where inhibition of actinomycin D-induced apoptosis was observed [102] or in macrophages from the cell line RAW 264.7 infected with *Leishmania major* where apoptosis diminished even in the presence of cycloheximide [103]. In neutrophils, it has been observed that spontaneous apoptosis is inhibited by *Leishmania major* due to a decrease in caspase-3 activity [104]. It has also been demonstrated that amastigotes and promastigotes of *Leishmania mexicana* inhibit camptothecin-induced apoptosis in monocyte-derived dendritic cells [105, 106]. Moreover, *Leishmania* parasites are characterized for presenting differences among different species and also intraspecific. In particular, it has been shown that different strains of *L. major* cause diverse clinical manifestations in susceptible BALB/c mice [107]. While the infection with the strains V1 and IR137 could be resolved, the infection with the LV39 strain presents a severe course of infection, which cannot be resolved. The infection of RAW 264.7 macrophages with the less virulent strains (V1 e IR37) of *L. major* showed a lower degree of inhibition of apoptosis as compared to the infection with the more virulent (LV39) [103].

6. Signaling pathways involved in the inhibition of apoptosis by *Leishmania*

As it has been just mentioned, *Leishmania* has the capacity to inhibit apoptosis of different cells; however, the mechanisms involved in this inhibition have not been fully deciphered. MAPK and PI3K have been implicated due to their participation in apoptosis and the intervention of *Leishmania* with these kinases. Regarding the role of *Leishmania* infection in the modulation of proapoptotic pathways such as MAPK, it has been shown that *L. mexicana* amastigotes and promastigotes significantly reduced MAPK JNK and p38 phosphorylation in monocyte-derived dendritic cells [108, 109]. Other authors working with the same species showed that the inhibitoy effect in the activation of MAPK in dendritic cells was only observable in immature dendritic cells since maturation driven by the stimulation with LPS did not suppress MAPK phosphorylation, in particular JNK [110]. In bone marrow macrophages (BMM), previously stimulated with IFN-γ, it was also shown that *L. donovani* promastigotes exerted a similar effect of inhibiting the activation of p38, JNK, and ERK that was directly associated with TNF-α production, which ensured the survival of the parasite [111]. Other authors also demonstrated that inhibition of p38 was associated with an increase in the number of infected macrophages and parasite survival [112]. Interestingly, not only the parasite but also some surface components such as gp63 have been shown to inhibit the apoptotic signaling of MAPK p38 [113]. Other studies have shown that *Leishmania* infection can also activate MAPK as demonstrated with the infection of neutrophils with *L. major* that caused the transient activation of ERK1/2, which delayed apoptosis and the pharmacological inhibition of ERK1/2 phosphorylation reversed the effect. Moreover, the infection of neutrophils with *L. major* led to the enhanced and sustainable expression of the antiapoptotic proteins Bcl-2 and Bfl-1. As downstream events, the release of cytochrome c from mitochondria and processing of caspase-6 were inhibited, as well as a reduced expression of FAS on the surface of neutrophils [114]. In BMM the infection with infected with *L. amazonensis* it has been observed that ERK 1/2 activation generates an epigenetic modification in the IL-10 locus, which results in a great induction of this cytokine in the infected macrophages [115]. Also, macrophages grown in the presence

of LPG show an altered production of IL-12 associated with ERK activation and signaling [116]. Other authors demonstrated that ERK 1/2 activation induced by *L. amazonensis* yielded a lesser expression of CD40 and IL-12 production in bone marrow derived dendritic cells, with the subsequent inhibition of dendritic cell maturation. Specific ERK 1/2 inhibition induced the production of NO which caused an increase in parasite death [117]. Interestingly, *Leishmania* infection not only intervenes with signaling pathways that induce apoptosis but also with pathways that promote survival as it has been shown with the infection of BMM with *L. major* and *L. pifanoi* promastigotes that promotes resistance to apoptosis through activation of PI3K/Akt. It was also demonstrated that Akt phosphorylates Bad, which in turn interacts with the 14–3-3 protein, inhibiting it and boosting the antiapoptotic action of Bcl-2 [118]. It has also been demonstrated that infection of monocyte-derived dendritic cells with *L. mexicana* amastigotes activated antiapoptotic signals, such as PI3K/Akt phosphorylation [108]. Recently, the participation of Akt in the inhibition of apoptosis by *Leishmania* has been more widely analyzed. The infection of BMM or RAW 264.7 with *L. donovani* promastigotes and treated both with a specific Akt inhibitor or a dominant negative construct diminished the antiapoptotic effect, increased the production of IL-12, and decreased the production of IL-10, which resulted in loss of parasite survival. It was shown that in infected cells FOXO-1, a transcriptional regulator of pro-apoptotic proteins, is found mainly in the cytoplasm. The transfection of cells with FOXO-1, constitutively active that cannot be phosphorylated Akt and thus remained sequestered in the nucleus, led to a reduction of the antiapoptotic effect in infected period. Also, it was observed that the activation of Akt, induced by the infection of macrophages with L. *donovani* promastigotes, causes the inactivation of GSK-3β (Glycogen synthase kinase 3 beta), which permits the release of β-catenin in order to initiate the transcription of antiapoptotic proteins. It was shown that in infected cells and transfected with the constituvely active construct for GSK-3β by silencing β-catenin there was a loss in mitochondrial membrane potential along with the activation of caspase-3 and production of IL-12 [119]. This was the first observation showing that the reversion of the antiapoptotic effect diminishes parasite survival, which suggests that the Akt pathway is a pivotal step in the modulation of the cellular machinery since *Leishmania* through the modulation of Akt is capable of activating antiapoptotic proteins, inhibiting pro-apoptotic and also inhibiting the production of IL-12 [119].

Continuing with the role of *Leishmania* in modulating antiapoptotic pathways it has been shown that in murine macrophages infected with *L. donovani* promastigotes and treated with the apoptosis inductor, actinomycin D, there was an increase in the mRNA levels and protein level of MCL-1. Interestingly, the silencing of MCL-1 in infected cells dampened the antiapoptotic effect in a similar way as the silencing of the transcription factor CREB, which diminished the expression of MCL-1 and increased the apoptosis of cells [120]. In a different study, several populations of macrophages (derived from peripheral blood, THP-1 and murine) were infected with *L. donovani* and the expression of Bcl-2 increased twice with respect to uninfected cells. Also, the use of specific inhibitors for Bcl-2 increased the level of NO, which diminished the parasite load of the cells. Interestingly, it was shown that in patients with visceral leishmaniasis, there was an increase in the expression of Bcl-2 and the levels of NO in serum were very low [121].

Recently, the receptor of programmed death 1 (PD-1) has been associated with the effect of inhibition of apoptosis in cells infected with *Leishmania.* The induction of apoptosis with H_2O_2

in BMM and RAW 264.7 cells increased the expression of PD-1, while the infection with *L. donovani* diminished it along with the induction of apoptosis. The activation of PD-1 pathway was found to negatively regulate the phosphorylation of pro-survival AKT, which was reversed during infection [122].

During the induction of apoptosis, reactive oxygen species (ROS) are produced; on the other hand, an overproduction of (ROS) induces apoptosis. The analysis of the effect of *Leishmania* in the modulation of ROS was analyzed in RAW 264.7 macrophages treated with H_2O_2 and infected with *L. donovani* promastigotes and was observed that the parasite did not affect the level of ROS and apoptosis was inhibited along with a decrease in caspase 3 and 7, which could be due to Suppressor Of Cytokine Signaling (SOCS) along with the activity of thioredoxin and tyrosine phosphatases. The silencing of SOCS genes diminished thioredoxin levels and increased apoptosis of cells [123]. Recently, De Souza-Vieira et al. demonstrated the activation of two PI3K isoforms, PI3K γ (ROS dependent) and PI3K δ (ROS independent) in neutrophils infected with *L. amazonensis*. The activation of these isoforms, in turn, activates the ERK pathway downstream, which is associated with the process of netosis with the subsequent activation of ROS and the release of neutrophil extracellular traps (NETs) [124].

7. Inhibition of apoptosis by *Trypanosoma cruzi*

7.1. Immune response to *T. cruzi*

Chagas' disease affects nearly 8 million people in Latin America [125] and is caused by the intracellular parasite *Trypanosoma cruzi*. The infection with *T. cruzi* is characterized by an acute phase that can be controlled by the immune system of the host. Afterwards, patients can remain asymptomatic or develop a chronic phase that affects mainly the heart and peripheral nervous system [126, 127]. In some cases, patients seem asymptomatic although they present several damages. *T. cruzi* has the capacity to infect virtually any cell where infective tripomastigotes reach the cytoplasm, replicate, lyse the cell and infect other cells. One of the cells inside the mammalian host where *T. cruzi* replicates is the macrophage. These cells are crucial for the immune response against the parasite because, depending on the stimulus, can be classically or alternatively activated. Classically activated macrophages (M1) produce nitric oxide (NO) that has the capacity of killing *T. cruzi*, whereas alternatively activated macrophages, belonging to the M2 spectrum, synthesize polyamines that actually promote infection [128, 129]. Thus, one of the most important mechanisms of protective immunity against *T. cruzi* is the classical activation of macrophages for the elimination of the intracellular parasites. *T. cruzi* must control the activation of macrophages and inhibit apoptosis in order to perpetuate inside the cells. To achieve this, parasites must reduce the production of toxic molecules, including NO and its derivatives [130, 131] and must escape from the parasitophorous vacuole [132].

The development of a specific immune response against *T. cruzi* overcomes the evasion strategies displayed by the parasite. Antibodies as well as T cells are required for the control of infection [133–135], where both CD4 and CD8 T cells produce IFN-γ that activates macrophages to restrain infection [136, 137], while CD8 T cells eliminate cells harboring parasites in the cytoplasm and also promote immunopathology in the heart [138, 139]. Some of these

mechanisms are regulated by cytokines, such as IL-10 and TGF-β, which diminish inflammation and thus pathology, but might contribute to the persistence of parasites [140, 141]. Also, it has been demonstrated that apoptosis of lymphocytes in the course of *T. cruzi* infection down regulates T-cell expansion [142, 143], B-cell response [144], parasite killing by M1 [143, 145], and CD8 T-cell-mediated immunity [146, 147]. Furthermore, infection is also promoted by the phagocytosis by macrophages of apoptotic T lymphocytes in a matter dependent of prostaglandins, TGF-*β*, and polyamine biosynthesis [148], which are characteristic of M2 activation [128]. In contrast, the blockade of prostaglandin production or the inhibition of T lymphocyte apoptosis by caspase inhibitors reduces parasite growth *in vitro* and parasitemia in an experimental model of Chagas disease [148, 149].

7.2. Apoptosis modulation in *T. cruzi* infection

7.2.1. Apoptosis induction

As just mentioned, it has been demonstrated that there is intense apoptosis of T lymphocytes during the course of *T. cruzi* infection [142]. The induction of apoptosis occurs through the extrinsic pathway as the infection with *T. cruzi* provokes the expression of both Fas (CD95) and Fas ligand (FasL) [143, 145], caspase-8 activity, and activation of effector caspase-3 [143, 144] in T lymphocytes from *T. cruzi*-infected mice. CD8 T lymphocytes help in the control of infection by *T. cruzi*, and the induction of apoptosis of these cells disrupts the immune response and interestingly affects macrophage activation. Apoptosis of CD8 T lymphocytes promotes macrophage differentiation toward an M2-like phenotype, which favors *T. cruzi* infection [150].

7.2.2. Apoptosis inhibition

T. cruzi resides in the cytoplasm of diverse cells, and thus CD8 lymphocytes are important for their elimination. It has been shown that CD8 T cells are preferential targets, as compared to CD4 lymphocytes, for early effects of apoptosis inhibition in acute infection [146, 151]. In addition to macrophages, *T. cruzi* infects cardiomyocytes and it is common to find an intense myocarditis during the acute phase of infection. Despite cardiac damage, infected individuals may remain asymptomatic for decades. Thus, *T. cruzi* may directly prevent cardiomyocyte death in order to prevent heart destruction and favor its survival. It has been shown that *T. cruzi*, as well as cruzipain, an important *T. cruzi* antigen, promotes survival of cardiomyocytes cultured under serum deprivation through the expression of the antiapoptotic protein Bcl-2, but not of Bcl-xL. Also, *T. cruzi* displays other antiapoptotic strategies such as the phosphorylation of Akt and ERK 1/2, which differentially modulate Bcl-2 family members [152]. In addition, cruzipain enhances arginase activity that favors parasite growth within the cell. Interestingly, the inhibition of arginase activity by NG-hydroxy-L-arginine (NOHA) abrogated the antiapoptotic action of cruzipain suggesting that arginase activity is required for the survival effect of cruzipain [153].

Apart from invading the heart, *T. cruzi* colonizes the peripheral nervous system and it has been shown that the infection of Schwann cells by *T. cruzi* suppresses host cell apoptosis caused by growth factor deprivation. The antiapoptotic effect of the parasite has been related to the interaction of Akt with *T. cruzi* PDNF, glycosylphosphatidylinositol (GPI)-anchored parasite-derived neurotrophic factor, known mostly for its neuraminidase and sialyltransferase activities [154].

8. Apoptosis inhibition in *Toxoplasma gondii* infection

Toxoplasma gondii is an obligate intracellular parasite capable to infect almost all types of nucleated cells and has developed multiple mechanisms to avoid immune elimination. It has been reported that this parasite can modulate multiple signaling pathways in their host cells in order to inhibit apoptosis, ensuring in this way its survival and persistence during infection. As a clear example of this process, different evidences have shown a failure in the activation of caspase 8, caspase 9, and caspase 3 after apoptosis induction in *T. gondii* infected cells [155–158].

The activation of the NF-κB transcription factor has been pointed as a pivotal mechanism used by *T. gondii* to inhibit apoptosis in several host cell types [157, 159, 160]. Following infection, it has been described that *T. gondii* induces NF-κB translocation into the nucleus, where this factor induces the transcriptional upregulation of genes that codify for antiapoptotic proteins that belong to the Bcl-2 and IAP families [157, 159, 160]. The translocation of NF-κB to the nucleus and subsequent gene transcription is clearly dependent of the host cell IκB kinase (IKK), which phosphorylates the IκB inhibitor molecules that maintain NF-κB inactive, allowing after this phosphorylation the activation and nuclear translocation of this transcription factor. However, in *T. gondii*-infected cells, the existence of a novel parasite-derived IκB kinase (TgKK) has been identified at the parasitophorous membrane together with phosphorylated IκB molecules [161]. In this sense, both host IKK and TgIKK cooperate for a continuous NF-κB activation during infection, in a process in which the host IKK could be inducing the initial phosphorylation of IκB molecules, followed by a sustained participation of the TgIKK, which presence increases as the parasite replicates [162].

Besides the NF-κB-dependent inhibition of apoptosis during infection with *T. gondii*, an inhibition of caspase 9 and caspase 3 activation through a direct blockage, exerted by the parasite, of apoptosome formation and cytochrome c release has been proposed [156, 162]. In this regard, the *T. gondii*-dependent inhibition of cytochrome c release from the mitochondria is associated with a parasite direct degradation of the proapoptotic proteins Bad and Bax, without affecting the mRNA levels for these proteins in the host cell, a fact that suggests that the parasite is able to block directly antiapoptotic proteins of the Bcl-2 family in a host cell transcription machinery-independent process [163, 164]. Additionally, among other signaling pathways hijacked by *T. gondii* to tilt the balance of the Bcl-2 family proteins toward an antiapoptotic state of the host cell during infection, the modulation of the PI3-K/PKB/Akt pathway has been involved [155, 165]. In *T. gondii*-infected macrophages, it has been documented that this parasite activates PI3-Kinase (PI3-K), which in turns phosphorylates protein kinase B (PKB/Akt), which acts as an apoptosis inhibitor of the host cells [165].

In addition to the inhibition of apoptosis by *T. gondii* via the mitochondrial pathway, this parasite has also been reported to mediate the inhibition of apoptosis through the extrinsic pathway [166]. In this regard, it has been demonstrated that during infection, *T. gondii* can inhibit Fas/CD95-triggered apoptosis in host cells by inducing an aberrant processing and degradation of the initiator caspase 8, a process that results in a decrease in the recruitment of this protease to the death-inducing signaling complex and the inability to activate effector caspases, for example, caspase 3 and caspase 7 [166].

Due to the fact that host defense in chronic infections due to *T. gondii* is critically dependent on the cytotoxic activity of CD8 T cells, which induce apoptosis of the infected cells via

the secretion of granzyme proteases, it is not surprising the blockage of this pathway by the parasite. Hence, *T. gondii* has been demonstrated to protect their host cells from Granzyme B-induced apoptosis, involving a mechanism by which the parasite abrogates the activity of Granzime B in the infected cells [167].

9. Apoptosis inhibition in *Plasmodium* spp. infection

In the mammalian host, *Plasmodium* parasites infect primarily hepatocytes and erythrocytes, and modulation of apoptosis by this parasite in both host cell types has been found to be crucial during infection. After transmission by the *Anopheles* mosquito, *Plasmodium* sporozoites are rapidly transported to the liver, where they invade and develop within hepatocytes before reaching erythrocytes [168]. In the liver, sporozoites transmigrate through the cytosol of multiple hepatocytes, causing wounding in the traversed cells with the release of the hepatocyte growth factor (HGF), which helps the parasite to reach a final hepatocyte in which it will reside and multiply [158, 169, 170]. It has been proposed that HGF binds to the c-mesenchymal-epithelial transition factor (c-Met) located on the surface of hepatocytes, a process that leads to PI3-K activation and a further protection of these cells from apoptosis [171, 172]. Albeit PI3-K activation trough HGF/c-Met signaling has been proposed to protect hepatocytes from apoptosis during early liver stages of infection with *Plasmodium*, other data suggest that PI3-K activation is not required to maintain this antiapoptotic state [173].

During the blood stage of infection with *Plasmodium*, in which merozoites invade erythrocytes, multiple changes are induced in the host cell by the parasite in order to satisfy its nutritional requirements [164]. One of these changes is the activation of Ca^{2+} permeable channels in the plasmatic membrane of erythrocytes and the posterior entry of Ca^{2+} into these cells. An increase in the intracellular concentration of Ca^{2+} in erythrocytes has been demonstrated to induce a type of programmed cell death called eryptosis, which is characterized by cell shrinkage, cell membrane blebbing, and exposure of phosphatidyl serine, resembling apoptosis [174]. Due that infection with *Plasmodium* leads to the entry of Ca^{2+} into the erythrocytes and that the increment of the concentration of this ion stimulates eryptosis, it has been shown that *Plasmodium* can delay the execution of this programmed cell death mechanism by sequestering free Ca^{2+} ions present in the cytosol of erythrocytes [175].

10. Conclusion

Both apoptosis and its inhibition are fundamental biological processes for the homeostasis of an organism. Both processes are present throughout life and are essential for growth, development, and reproduction. Studies on the molecular mechanisms that inhibit apoptosis have been carried out in order to elucidate the specific signaling pathways that take place during apoptosis inhibition. Up to date, various routes implicated in apoptosis activation or inhibition have been rooted out; however, there is still much to be found. Ironically, the same pathways that are involved in homeostasis and health participate in cell death processes that occur during infections and function as a defense mechanism

against intracellular pathogens. In counterpart, microorganisms have developed a wide array of strategies to evade apoptosis of their host cell. Some of these strategies involve the hijacking of signaling pathways that participate in apoptosis. The better understanding and gaining of knowledge on these intracellular circuits and the physiopathology behind them will permit the development of new strategies and drugs to effectively treat the pertaining diseases mentioned in this work.

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Author details

Laila Gutiérrez-Kobeh[1]*, Jorge Rodríguez González[1,3], Rosalino Vázquez-López[2] and Arturo Wilkins-Rodríguez[1]

*Address all correspondence to: lgutierr@unam.mx

1 Research Unit UNAM-INC, Research Division, School of Medicine, National Autonomous University of Mexico-National Institute of Cardiology Research Division, Mexico City, Mexico.

2 Microbiology Department, CICSA, Health Science Faculty, Universidad Anáhuac México Norte, Huixquilucan, México

3 Posgrado en Ciencias Biológicas, Facultad de Medicina, Unidad de Posgrado, UNAM, Ciudad Universitaria, Ciudad de México, México

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Apoptosis at Resistance to Chemotherapy and at Electromagnetic Radiation

Role of Apoptosis in Cancer Resistance to Chemotherapy

Lichan Chen, Yanyun Zeng and Shu-Feng Zhou

Additional information is available at the end of the chapter

Abstract

Cancer is a leading cause of death in human beings. Surgery, chemotherapy, radiotherapy, immunotherapy, and biologically targeted therapy are common modalities for cancer treatment. However, cancer resistance is common in chemotherapy and often leads to therapeutic failure. This chapter addresses the role of apoptosis in tumor's resistance to chemotherapy and the underlying mechanisms. Cancer cells are always resistant to apoptotic signals via a series of biochemical changes. Cancer cells are resistant to chemotherapeutic agents that are potent apoptosis inducers via multiple mechanisms, such as upregulated anti-apoptotic signals and downregulated pro-apoptotic signals, faulty apoptotic signaling, faulty apoptosis initiation and implementation, etc. We also discuss the possible approaches to overcoming cancer resistance to chemotherapy due to altered apoptosis.

Keywords: apoptosis, cancer resistance, chemotherapy

1. Chemotherapy and cancer resistance: fact, evidence, and outcome

Cancer is a major public health problem. According to the International Agency for Research on Cancer (IARC), about 14.1 million new cancer cases were reported in 2012 worldwide, and 8 million occurred in developing countries [1]. Cancer ranks second among the leading causes of death in the United States. US Final Mortality Data (2015) showed that lung cancer is the first leading cause of death in all ages with 5-year survival rate around 18% [2]. The main reason for high mortality is that most cancers are difficult to be diagnosed by routine examinations in the early stage, due to the slight change in the tumor biomarker level and the

unapparent symptoms. Fortunately, benefiting from the advances in cancer treatment and the alteration in personal habits (e.g. reduction of smoking), cancer mortality has been declining over the past two decades [2].

Among cancer treatments, chemotherapy is one of the most effective modalities. The patients are given first-line treatments after the clinical diagnosis. With the promising and wide spectrum of anticancer effects, the first-line drugs are very likely to kill the cancer cells and increase survival rate among patients. However, some patients may suffer relapse or cancer metastasis, and second-line treatments come to stage. Chemotherapy usually uses alkylate and anthracyclines as antimetabolic agents in clinical treatments. These chemotherapeutic drugs mainly take effect through activation of caspases and calcium-dependent nucleases to induce cancer cell apoptosis. For example, taxol, a microtubule inhibitor, promotes cancer cell apoptosis by inhibiting phosphorylation of apoptotic protein Bcl-2 [3], while glucocorticoid, the chemotherapeutic drug for acute lymphoblastic leukemia, induces apoptosis by regulating a sequence of apoptosis-related genes in malignant cells [4]. The chemotherapeutic drugs can also transfer the pro-apoptotic signals to cancer cells, ending the cell cycle and programing cell death.

However, cancer resistance is common in chemotherapy and leads to therapeutic failure. Cancer resistance can be sorted into primary resistance and acquired resistance. The primary resistance originates from the natural immunity, while the acquired resistance is gained and developed during treatment. Drug resistance can be caused by changing drug targets. For example, DNA-targeting drugs take effect in the nucleus; however, the drugs would disperse into the cytoplasm in the presence of a non-ABC transporter [5]. As a result, the chemotherapeutic drugs fail to target DNA in the nucleus, but accumulate in the extracellular environment. In addition, patients would develop drug resistance after long exposure to the same agent, and may even develop cross-resistance to non-related drugs and multidrug resistance (MDR). The mechanism of drug resistance is intricate, involving the alteration of transporter pump, the aversion of apoptosis and autophagy, the mutation and amplification of oncogenes and tumor suppressor genes, the variation of drug metabolism, etc. To remedy cancer resistance, researchers have tried many solutions. For instance, Wang et al. have used gambogic acid (GA) as an auxiliary to remedy doxorubicin (DOX) resistance in breast cancer [6, 7]. GA could reduce the expression of P-glycoprotein (P-gp), a key protein in DOX resistance, and promote the accumulation of DOX in cancer cells [6]. Furthermore, GA has been reported to induce apoptosis via p38 MAPK pathway. GA increases the apoptotic rate by downregulating the expression of survivin mRNA [6]. Even though the mechanism of the combined treatment is still unclear, it seems to be a promising approach for DOX resistance in breast cancer. To further improve the efficacy of chemotherapy, the mechanism of cancer resistance should be fathomed.

2. Mechanisms of cancer cells evading apoptosis

Apoptosis is an autonomous process that involves the activation, expression, and regulation of a wide range of genes, leading to programed cell death to remove unwanted or abnormal cells in organisms and maintaining a stable internal environment. Apoptosis mediates the

programed cell death either in a caspase-dependent or in a caspase-independent pathway. The caspase-dependent pathway can be classified into the extrinsic pathway and the intrinsic pathway, as illustrated in **Figure 1**. Caspases, a cysteine protease family, can be divided into the apoptotic subfamily and the inflammatory subfamily according to the pathway they involve. Among the known 18 mammalian caspases, caspases 2, 3, 6, 7, 8, 9, and 10 can be categorized as apoptotic caspases: caspase 2 is involved in various cell death pathways; caspases 3, 6, and 7 work as apoptotic executors, while caspases 8 and 10 are essential in the extrinsic pathway and caspase 9 is essential in intrinsic pathway. As shown in **Figure 1**, the extrinsic pathway facilitates apoptosis by activating caspases through the death receptor ligands on the cell surface. The death receptor ligands are closely related to the tumor necrosis factor (TNF) receptor superfamily, including the TNF-related apoptosis-inducing ligand (TRAIL), TNFR1 (CD120a), Fas (APO-1/CD95), Weasl (APO-2/DR3), TRAIL-R1 (DR4), TRAIL-R2 (DR5), and DR6. Take Fas as an example. Fas/FasL is one of the well-known death receptors associated with signaling pathways in immune and pro-apoptotic effect [8]. The Fas exists in two forms: membrane Fas (mFas) and soluble Fas (sFas). mFas and sFas bind to FasL in a competitive way. The binding of mFas and FasL induces pro-apoptosis, while the binding of sFas and FasL has no similar effect. With the binding of mFas and FasL, mFas-associated death domain
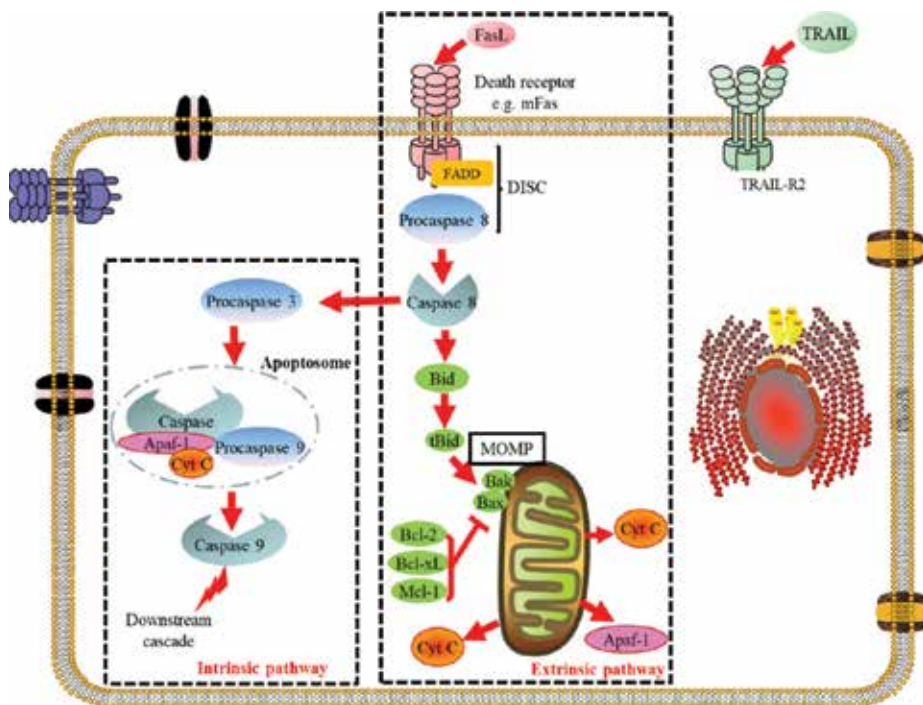
Figure 1. The intrinsic and extrinsic apoptotic pathways. The extrinsic pathway facilitates apoptosis by activating caspases through the death receptor ligands (e.g. mFas) on the cell surface. With the binding of mFas and FasL, mFas-associated death domain (FADD) combines with procaspases 8 and 10, leading to the formation of death-inducing signaling complex (DISC) which activates the downstream signal cascade. MAC forms on the mitochondrial outer membrane and releases cytochrome C into the cytosol. The intrinsic pathway of apoptosis is initiated by cytochrome C released from mitochondria to the cytosol. In the presence of ATP/dATP, cytochrome C interacts with the apoptotic protease-activating factor (Apaf-1) to promote the formation of apoptosome with procaspase 9.

(FADD) combines with procaspases 8 and 10, leading to the formation of death-inducing signaling complex (DISC), which activates the downstream signal cascade [9]. The activated caspase 8 modifies Bid into tBid. tBid binds with Bak and Bax, which are pro-apoptosis proteins that control the permeability of mitochondrial outer membrane, to form mitochondrial apoptosis-induced channel (MAC). The intrinsic pathway of apoptosis, also known as the mitochondrial pathway, is initiated by cytochrome C. Cytochrome C is a key protein for electron transfer in mitochondria. Mitochondria releases cytochrome C into the cytosol through MAC in response to stresses of apoptosis-inducing factors [9]. In the presence of ATP/dATP, cytochrome C interacts with the apoptotic protease-activating factor (Apaf-1) in the cytosol to form a complex and promotes the formation of apoptosome that activates procaspase 9 [9]. The activated caspase 9 further activates the downstream caspases.

Notably, a class of proteins exerts anti-apoptosis and pro-apoptosis effects in the apoptosis pathway. These proteins include the Bcl-2 family and inhibitors of apoptosis proteins (IAPs). The Bcl-2 protein family can be classified into two functional groups—one of which has an inhibitory effect on apoptosis through inhibition of MAC formation, such as Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Ced-9, while the other has a promoting effect on apoptosis by promotion of MAC formation, such as Bax, Bak, Bik, Bid, and Harakiri [10]. IAPs are the family of caspase inhibitors, including survivin, livin, Bruce (Apollon), cIAP1, cIAP2, IAP-like protein 2 (ILP-2), the X-linked inhibitor of apoptosis protein (XIAP), and neuronal apoptosis inhibitory protein (NAIP) [11]. Obviously, the homeostasis between anti-apoptosis proteins and pro-apoptosis proteins is essential for cell survival.

In addition to the extrinsic pathway and the intrinsic pathway, there also exists caspase-independent pathway. This pathway relies on apoptosis-inducing factors (AIFs). AIFs are flavoproteins present in the inner mitochondrial membrane [12], and exhibit the pro-apoptosis effect. AIFs are released into cytoplasm along with the increased permeability or the cleavage of mitochondria. Then, AIFs enter the nucleus and lead to chromatin condensation and break into fragments. Polster has studied the relationship of AIFs and mitochondrial ROS production [13]. Insufficient AIF would reduce the electron transport chain complex I, which relates to chronic neurodegeneration [14].

The cancer cells evade apoptosis via various mechanisms. Theoretically, in order to resist apoptosis, cancer cells would upregulate anti-apoptotic signals (e.g. Bcl-2, Akt, Mcl-1, etc.) and downregulate pro-apoptotic signals (e.g. Bax, Bak, Bad, etc.), initiate and implicate faulty apoptosis, etc. The detail is discussed below.

2.1. Cancer cells resisting pro-apoptotic signals

In human cancer cells, the downregulation of pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bim, etc.) and the upregulation of anti-apoptotic proteins (e.g. Bcl-2, Akt, Mcl-1, etc.) hinder the formation of MAC, inhibiting the release of cytochrome C from mitochondria and leading to the immortal character of the cancer cells. For example, the increased ubiquitination level of Bax has been found to be positively correlated to tumor malignant degree [15]. The decreased expression of Bad has been observed in small-cell lung cancers (SCLC), breast carcinoma, and gastric cancer. Furthermore, cancer cells regulate Bim in the pro-transcriptional,

transcriptional, and post-translational levels to disturb the interaction between Bim and Bak/Bax, and thus change the mitochondria's outer membrane permeabilization (MOMP). Overexpression of Bcl-2 inhibits cell death induced by a variety of cytotoxins, and enhances cell resistance to DNA damage factors and most chemotherapeutic drugs [16]. Bcl-2 has been shown to inhibit p53-mediated apoptosis but cannot inhibit p53 translocation toward nucleolus or p53-mediated growth arrest. The possible role of Bcl-2 is to block the activation of the apoptotic signals to their target molecules.

In addition, the abnormal expression of IAPs in cancer cells also increases cancer malignancies. The overexpression of IAPs abolishes the downstream caspase cascade. IAPs form a complex with the baculovirus-IAP repeat (BIR) domain of caspases, inhibiting the catalytic activity of caspases 3, 7, and 9 and blocking the process of apoptosis. cIAP1 inhibits apoptosis by binding to the BIR2 domain, which is used for activating caspases 3 and 7, resulting in ubiquitin-mediated proteasomal degradation. Werner et al. [17] have reported that upregulation of cIAP1/2 inhibits TRAIL-mediated apoptosis in follicular thyroid cancer. The IAPs have also been reported in interaction with NF-κB [18]. They are key molecules that regulate tumor cell apoptosis and chemo-sensitivity, developing new targets for reversing tumor cell resistance and improving treatment efficacy.

2.2. Cancer cells reducing anti-apoptosis signals

A variety of signal pathways are involved in the anti-apoptosis process. The TNF family is associated with apoptosis and malignant tumorigenesis. It has been reported that the translational level of Fas is downregulated in prostate cancer and liver cancer. TRAIL, another membrane of TNF family, intrigues wide anticancer effect by exerting pattern-like function of mFas. It has been found that some cancers show primary resistance and even develop multiple-mechanism resistance to TRAIL-induced apoptosis [19]. For example, overexpression of TRAIL receptor 3 (TRAIL-R3/DcR1) and TRAIL receptor 4 (TRAIL-R4/DcR2) is considered to contribute to the TRAIL-mediated apoptosis evasion of cancer cells. TRAIL-R3 and TRAIL-R4 are decoy receptors without intercellular death domain. The incapability of TRAIL-3 and TRAIL-4 to associate with procaspases 8 and 10 to form DISC attenuates the activation of downstream signaling pathway [20]. Furthermore, gene mutation of diverse proteins generates the anti-apoptotic effect. Shlyakhtina Y. and his colleagues [21] have studied TRAIL-R2 (DR5) within isogenic cancer cell populations. The models were pretreated with distinctive inhibitors, and the results showed that apoptosis evasion involves kinase cascades of functional Erk1/2, p38, and Akt.

Alteration of the p53 pathway also contributes to apoptosis evasion. The p53 gene is a human tumor suppressor gene. The p53 protein endows anticancer effect by activating defected gene repair and causing apoptosis of cancer cells if the damage is irreparable. p53 regulates apoptosis through Bax/Bcl-2, Fas/Apol, IGF-BP3, and other proteins. Inactivation, elimination, and abnormal expression of the p53 gene play important roles in tumorigenesis. About 80% of human tumors are caused by dysfunctional p53 signaling and 50% by p53 gene mutation [22]. Abnormal expression of p53 downregulates Bax/Noxa/Puma expression and upregulates Bcl-2. The upregulation of Bcl-2 prevents cytochrome C release from the mitochondria, inhibiting p53-mediated apoptosis. The downregulation of Bax prevents the formation of MAC on the

outer membrane of mitochondria, reducing the pro-apoptotic effect [23]. Furthermore, mouse double minute 2 homolog (MDM2) has also been found to play a pivotal role in the inhibition of p53-mediated apoptosis by negative regulation. In cancer cells, the increase of MDM2 transcription and p53 ubiquitination attenuates p53-mediated apoptosis [24].

NF-κB pathway, one of the highly conserved signal pathways of activating gene transcription, takes complicated apoptotic effects in different cells. Activated NF-κB improves the transcription level of survivin, Bcl-2, Bcl-X_L, and XIAP, resulting in resistance to the chemotherapeutic pro-apoptotic signals [5]. However, NF-κB renders pro-apoptotic effect through upregulating caspase 4 in Fas-induced neuroblastoma cell apoptosis [25]. In addition, NF-κB upregulates pro-survival genes via Akt activation.

PI3/AKT pathway mediates the survival signals in cancer cells. Akt is correlated to phosphorylation of diverse signal molecules and has a profound effect on cell survival, cell cycle progression, cell growth, and metabolism. The overexpression and overactivation of Akt have been observed in malignant tumors. For example, Zheng [5] has revealed that the paclitaxel-resistance developed in NSCLC can be ascribed to Akt-1 overexpression and Akt-2 gene amplification. In addition, Akt promotes the phosphorylation of Bad on Ser136/Ser112, leading to the suppression of apoptosis [26]. Akt phosphorylates Forkhead-box Class O (FoxO), a protein family governing a line of apoptotic gene transcription in PI3K/Akt pathway. The phosphorylated FoxO binds with 14-3-3, stays in the cytoplasm, and fails to execute transcription in nucleus [27].

2.3. Abnormal cross talk of autophagy and apoptosis

Autophagy is the process of self-digestion and degradation of proteins, organelles, and cell to obtain essential elements and energy for cell survival. Under normal physiological conditions, autophagy allows the cells to maintain homeostasis by transporting damaged or senescent substances into the lysosome, preventing the intercellular accumulation of toxic or carcinogenic substances and inhibiting cell carcinogenesis. However, in the tumor microenvironment, autophagy supplies nutrients to cancer cells and promotes tumor growth. The cross talk between autophagy and apoptosis contributes to cell viability (**Figure 2**). Apoptosis regulates autophagy either through specific apoptotic protein regulation or by caspase activation, while autophagy regulates apoptosis through: (1) specific autophagy protein regulation; (2) caspase activation (autophagosome required); (3) autophagic degradation (both autophagosome and lysosome required) and mutual signal pathways [28].

The abnormal apoptosis-autophagy cross talk helps cell death evasion. First, the unusual autophagy proteins would result in apoptosis evasion. In normal apoptotic cells, autophagosome with the regulation of autophagy protein 9 (ATG9), ATG16L1, ATG5, and ATG12 shows a pro-apoptotic effect. However, in abnormal apoptotic cells, less ATG5 translocation and interaction with Bcl-X_L in mitochondria reduces cytochrome C release; meanwhile, the dwindling binding of ATG12 to Bcl-2 and Mcl-1 decreases caspase activation [28]. In addition, the Beclin-1 interacting UV radiation resistance-associated gene (UVRAG) shows an inhibitory effect on apoptosis by binding to Bax [29]. Second, aberrant activations of caspases lead to the longevity of cancer cells. For example, caspase 8 is recruited by autophagosome, and caspase 8/RIPK1 is important for apoptosis-autophagy cross talk [28]. The inefficient activated
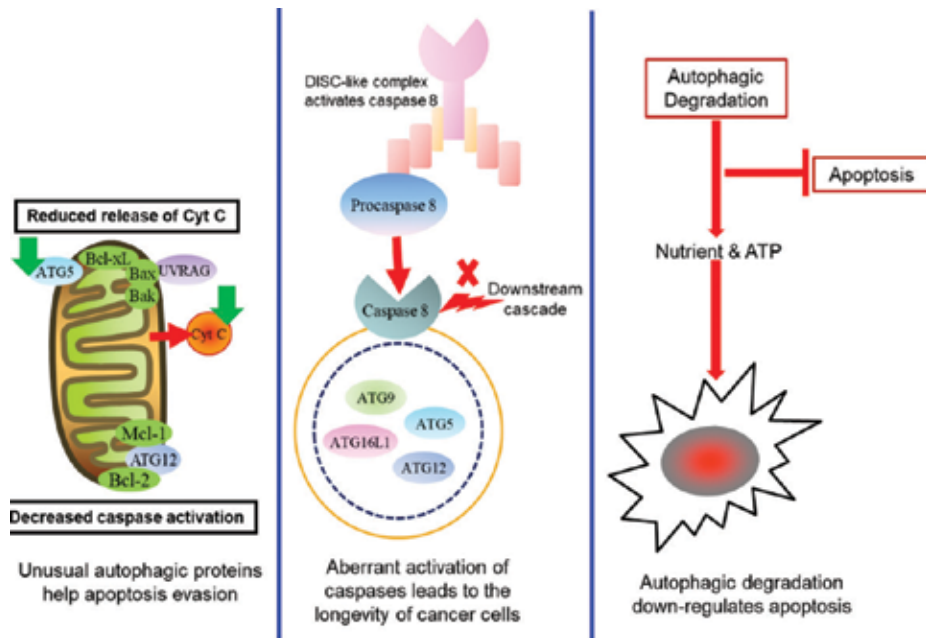
Figure 2. Abnormal apoptosis-autophagy cross talk. (1) The unusual autophagy proteins would result in apoptosis evasion. In abnormal apoptotic cells, fewer ATG5 translocate and interact with Bcl-X$_L$ in mitochondria, reducing the release of cytochrome C; meanwhile, the dwindling ATG12 binds to Bcl-2 and Mcl-1 results in the decrease of caspase activation. (2) Aberrant activation of caspases leads to cell immortality. For example, caspase 8 activated by DISC-like complex is recruited by autophagosome. (3) Autophagic degradation downregulates apoptosis.

caspase 8 may fail to trigger the downstream cascade of apoptosis. Furthermore, Beclin-1 is mediated by caspases; mutant D133A+D146A Beclin-1 has been reported to be resistant to chemotherapy [29]. Third, autophagic degradation downregulates apoptosis. In normal condition, starving cells accelerate apoptosis. However, the apoptosis is attenuated in tumors because the neighboring cancer cells degraded by autophagy provide nutrient and ATP for tumorigenesis. Fourth, malfunctioned signal pathways hinder the cell death process. The p53 protein can be regulated by AMPK pathway and degraded by chaperone-mediated autophagy. In p53-induced apoptosis, the downregulation of damage-regulated autophagy modulator (DRAM) mRNA has been observed in tumor with wild-type; the deficiency of DRAM promotes cell survival [30]. Han et al. [31] have reported that suberoylanilide hydroxamic acid (SAHA) may promote autophagy by stimulating TRAIL-R2-CTSB via AKT pathway.

3. Possible approaches to overcoming cancer resistance

In clinical treatments, the principle to treat drug-resistant cancer is targeting the specific target with the coordinated agent. However, the strategy is idealized since drug-resistant cancer usually involves multiple signaling pathways as well as multiple targets, and the contributions of each target is hard to be calculated. Therefore, treating drug-resistant cancer with the drugs against

the wide-array targets would be more realistic. Similarly, drugs that would induce multiple cell death pathways are likely to circumvent cancer resistance to chemotherapy. Targeting drug transporter protein would be an effective approach to overcome cancer resistance to chemotherapy.

Combined therapies are also considered to be a possible way to overcome cancer resistance to chemotherapy. An ongoing clinical study, led by Okonogi and his colleagues [32], applied maximum dose of carbon ion radiotherapy (C-ion RT) with concurrent chemotherapeutic drugs in uterine cervical carcinoma. The overall survival rate of 31 patients with recommended dose (RD) treatment is 88%; only 2 patients suffered from gastrointestinal toxicities. The studies still require developing a better drug delivery method for longer treatment duration and larger crowd of patients.

Taking advantages of the advance in material science and nanotechnology, nanomedicine delivery systems show a promising potential to guarantee the efficacy of chemotherapy. For example, cerium oxide nanoparticles (CNPs) have been used as carriers to deliver curcumins [33]. The nanoscale delivery systems maintain the stability of curcumins in alkalescency environment and exert anticancer effects. The treatment with nanomedicines increases ROS accumulation and decreases the ratio of Bcl-2/Bax in human neuroblastoma cells, improving the therapeutic efficacy. Zhang et al. [34] used Dox-loaded DNA tetrahedron to target folate receptors in HT-29 colon cancer cells. The treatment efficacy was also enhanced.

4. Conclusions

Apoptosis is an essential process for the growth and development of organisms, while cancer cells obtain immortality by escaping programed cell death. Understanding the underlying mechanism of cancer resistance to chemotherapy is fundamental for efficient cancer treatment agents. In this chapter, we have discussed the mechanisms of cancer cells evading apoptosis, including downregulation of pro-apoptotic signals, upregulation of anti-apoptotic signals and abnormal cross talk of autophagy and apoptosis. Chemotherapeutic drugs induce pro-apoptosis in cancer cells; however, the upregulation of anti-apoptotic proteins, e.g. Bcl-2 and IAPs, would cause cancer resistance. Death receptors including NF-κB-, PI3/AKT-, and p53-related signaling pathways are also involved in the chemoresistance. Additionally, the aberrant autophagy may cause apoptosis evasion as well through autophagic protein regulation, caspase activation, and autophagic degradation. A further, in-depth understanding of apoptosis evasion would be helpful for developing strategies to circumvent cancer resistant to chemotherapy. Combined chemotherapeutic treatment, drugs targeting multiple targets, and using nanoscale drug delivery (nanomedicine) show promising potentials to overcome chemoresistance and achieve precision therapy.

Acronyms and abbreviations

AIF   Apoptosis-inducing factor

Apaf-1   Apoptotic protease-activating factor

ATG9	Autophagy protein 9
DISC	Death-inducing signaling complex
DOX	Doxorubicin
FoxO	Forkhead-box class O
GA	Gambogic acid
IAP	Inhibitors of apoptosis protein
MDM2	Mouse double minute 2 homolog
MDR	Multidrug resistance
NSCLC	Non-small cell lung cancer
P-gp	P-glycoprotein
TOP-1	Topoisomerase-1
TNF	Tumor necrosis factor
BH3-only protein	Bcl-2 homology domain only protein
DRAM	Downregulated damage-regulated autophagy modulator
MAC	Mitochondrial apoptosis-induced channel
UVRAG	UV radiation resistance-associated gene
MOMP	Mitochondria outer membrane permeabilization

Author details

Lichan Chen, Yanyun Zeng and Shu-Feng Zhou*

*Address all correspondence to: szhou@hqu.edu.cn

Department of Bioengineering and Biotechnology, College of Chemical Engineering, Huaqiao University, Xiamen, Fujian, China

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Electromagnetic Radiation from Cellphone Towers: A Potential Health Hazard for Birds, Bees, and Humans

Chanda Siddoo-Atwal

Additional information is available at the end of the chapter

Abstract

Microwave sickness syndrome was first identified in the 1950s by Soviet researchers. Symptoms included headache, fatigue, ocular dysfunction, dizziness, and sleep disorders. The main clinical manifestations were dermographism, tumors, blood changes, reproductive and cardiovascular abnormalities, depression, irritability, and memory impairment. Later in the 1970s, American researchers reported similar findings. Electromagnetic radiation (EMR) from modern cellphone towers is largely comprised of high-frequency radio waves or microwaves. The adverse biological effects of EMR from cellphone towers have been observed in birds, bees, and humans. The associated decline in fruit-eating seed dispersers such as wild birds and in insect pollinators such as bees could have serious consequences for human food production. In addition to noting this possible indirect effect of microwave radiation, a direct effect on human health was evaluated. According to a new approach to cancer risk assessment, based on an apoptotic model of carcinogenesis, it was determined that proximity to EMR from cellphone towers may pose a potential cancer risk in humans since microwave radiation can induce various apoptotic pathways leading to cell death in transformed human cell lines. The stimulation of cellular apoptosis resulting in deregulated cell proliferation is being increasingly linked to cancer and may provide a possible mechanism for microwave radiation carcinogenesis.

Keywords: electromagnetic radiation, microwave radiation, radiofrequency radiation, microwave radiation carcinogenesis, apoptosis

1. Introduction

The electromagnetic spectrum consists of ionizing and nonionizing radiation. Ionizing radiation includes ultraviolet (UV) rays, X-rays, and gamma (ϒ) rays. Electromagnetic radiation

(EMR) from cellphone towers is largely comprised of high-frequency radio waves or micro-waves. Microwaves lie in the nonionizing radiation portion of the electromagnetic spectrum which includes low-frequency (computers, power lines), medium-frequency (television, radio), and high-frequency (microwaves, mobile devices) radio waves (**Figure 1**).

Radio-frequency radiation (RFR) is emitted at varying frequencies by cellphone towers, cell phones, computers, Wi-Fi, microwave ovens, and other electronic devices. RFR frequency ranges between 10 KHz and 300 GHz. On average, Wi-Fi applications and microwave ovens utilize 2450 MHz. Cellphone technology uses transmission signals between 800 MHz and 3 GHz, while cellphone towers typically operate at 1900 MHz [1].

The adverse biological effects of EMR from cellphone towers have been reported in frugivores such as potential fruit-eating seed dispersers (birds), insect pollinators (bees), and humans. A decline in sparrow populations and other wild birds has been observed in the vicinity of cellphone towers in India. EMR from similar sources has also been correlated with decreased egg production in honey bees. In fact, in India, the problem is perceived as being sufficiently serious that a panel of scientists has recommended regular auditing of EMR levels and that EMR be recognized as a pollutant. They have also suggested implementation of a special law to protect urban flora and fauna from its effects [2–4].

The significant radioisotopes released as a result of the Chernobyl nuclear reactor explosion were iodine-131, cesium-137, strontium-90, and plutonium-241. Some of these radioisotopes
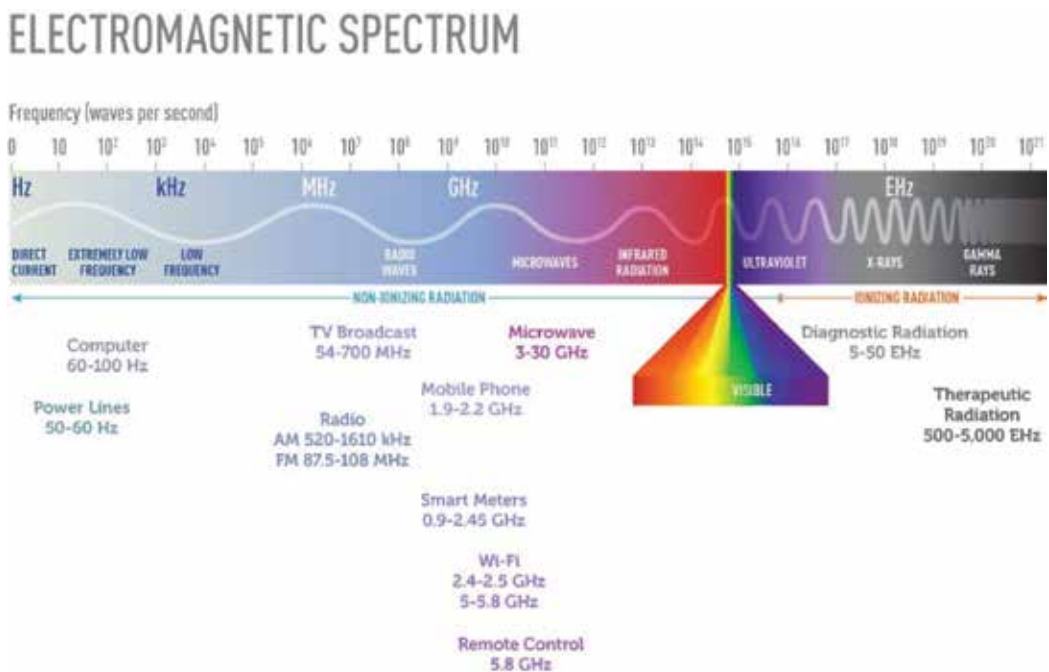
Figure 1. National Cancer Institute – National Institute of Health; 2016.

such as cesium-137 are emitters of gamma radiation. Cesium is one of the radioactive fission products routinely produced by a nuclear reactor during its operation. However, following the Chernobyl event, forest food products in surrounding regions of Europe were found to contain the highest recorded levels of the radioisotope cesium-137. For example, high contamination of reindeer meat was reported in Scandinavia. Pollen data from forests in the Bavarian Alps also show that radiocesium uptake by mosses uniquely reference the Chernobyl incident [5].

Previously, a local reduction in pollinators like bumblebees in the vicinity of Chernobyl has been linked to the production of fewer fruit and stunted fruit trees in highly radioactive areas. Moreover, a direct link has been established between radiation, pollinators, fruit abundance, and an abundance of frugivores such as fruit-eating seed dispersers [6]. Therefore, due to the observed reduction in wild birds and honey bees, it is possible that EMR may negatively impact fruit production, which is an important food crop, in areas of high cell tower concentration. It has already been reported that EMR from cell towers can affect the overall growth of agricultural crops and plants by reducing yield [2]. Since growing certain fruits rich in micronutrients may be beneficial in helping to prevent cancer, such a decline in crop yield could represent a significant loss for the agricultural industry as the demand for these phytochemicals like the polyphenols found in grape skin and grape seeds grows (resveratrol and procyanidins) [7].

In addition to this indirect effect on human health, the potential for a direct effect of EMR on human health was investigated. The adverse health effects of EMR from cell base stations and other cellular infrastructure are certainly contentious. Epidemiological data are lacking in this area and, at times, it is contradictory. Exposure levels are often difficult to quantify due to background EMR including from cell phones, computers, Wi-Fi, microwave ovens, and other electronic household devices. However, there is some research that suggests a degree of caution should be exercised in the installation of such cell base stations. Moreover, substantial evidence relating to microwave radiation exposure exists.

Microwave sickness syndrome was first identified in the 1950s by Soviet medical researchers. Symptoms included headache, fatigue, ocular dysfunction, dizziness, and sleep disorders. Clinically, dermographism, tumors, blood changes, reproductive and cardiovascular abnormalities, depression, irritability, and memory impairment were reported. Although the syndrome is reversible in its early stages, it is considered to be lethal over time [8].

Later American researchers found symptoms to include eczema, psoriasis, and allergic and inflammatory reactions in staff stationed at the US Embassy in Moscow, which the Soviet government irradiated secretly over a period of approximately 20 years. It is of interest that the power densities of the microwaves employed by the Soviets were comparable to modern cellphone base stations. They also observed neurological problems in males, reproductive problems in females, tumor increases (benign in men, malignant in women), hematological alterations, effects on mood and well-being, and eye problems. The average exposure time for each individual was between 2 and 4 years [9].

Despite these observed effects and other existing data, no satisfactory explanation for tumor formation based on classical experimental carcinogenesis models has been available so far.

Such traditional models rely heavily on DNA damage and the subsequent clonal expansion of mutated cells for their modality. In the past, it has been stated that no mechanism is known to cause cancer in the nonionizing radiation or radiofrequency radiation part of the electromagnetic spectrum since it does not damage DNA or cells directly like ionizing radiation [10]. Thus, it has been largely dismissed as a putative cause of cancer. However, recently, it has become apparent that the pathogenesis of cancer is closely connected with aberrantly regulated apoptotic cell death and the resulting deregulation of cell proliferation. A mechanism for gamma-radiation carcinogenesis based on an apoptotic model has already been proposed [11]. According to a new approach to cancer risk assessment, it was determined that EMR from cellphone towers may pose a cancer risk in humans since microwaves can stimulate p53-mediated caspase-3 activation and cell death in a human brain glioblastoma cell line [12] and Fas-induced and ERK-mediated apoptosis in human lymphocyte cell lines [13, 14].

2. Epidemiology

Aside from the Moscow study of the US Embassy staff, early epidemiological data were gathered from technically trained US naval officers routinely exposed to radar by Robinette et al. [15]. Radar transmission generates electromagnetic waves in the microwave domain. Interestingly, both these groups showed an elevated incidence of leukemia. In another large Polish study, military personnel exposed to radiofrequency microwave radiation from radio and radar showed very significant elevations in leukemia and other cancers [16].

The first epidemiological studies on populations living near cell base stations focused mainly on cognitive changes and neurobehavioral effects and started being conducted in 2002 [17]. However, an early Egyptian cellphone tower study in the Algharbia governate area (1999–2002) suggested an increase in the overall cancer rate of the local population by 7.5% [18]. Later, a German study found elevated cancer incidence in patients who had lived 5–10 years within 400 meters of a cell installation [19]. Another Israeli study indicated an association between increased cancer incidence and living in proximity to a cell base station [20].

There also appears to be a significant body of evidence suggesting that cell phones, which use EMR in the microwave range, can cause brain tumors and disturb brain function [21, 22]. One Swedish study reported that cellphone radiation increases the human brain tumor rate by 2.5 times [23]. In fact, in his extensive review on the subject, Cherry concludes that over 40 studies have revealed adverse biological or human health effects specifically from cellphone radiation and that there is extremely strong evidence that cell sites are risk factors for brain tumors and leukemia [24].

It should be noted that children may be more susceptible to damage from cellphone radiation since their bodies are still developing. There is epidemiological evidence to suggest that children are susceptible to leukemia from high power voltage (HPV) lines which emit low-frequency radio waves [25, 26]. Although no epidemiological data seem to have been collected

in children regarding exposure to high-frequency radio waves, there are reports that cellphone radiation penetrates deeper into the head of children and that certain tissues of the head like the bone marrow and eye absorb more radiation than in adults [27–29]. Specific absorption rate (SAR) is the term used to describe the absorption of RFR in the body and represents the rate of energy absorbed by a unit of tissue.

3. Animal studies

In vivo animal studies have demonstrated that potentially genotoxic effects in male Wistar rats following microwave exposure include the induction of micronuclei, an increase in the production of reactive oxygen species (ROS) which can trigger cellular apoptosis, and increases in various antioxidant enzyme activities like serum glutathione peroxidase, superoxide dismutase, and catalase [2]. Rats exposed to microwaves display a significant reduction in splenic activity of natural killer cells, which may help to provide host defense against the development of tumors [30]. Other cellphone radiation research in animals has shown that it doubles the cancer rate in mice [31]. Also, EMR from cellphones can increase mouse tumor necrosis factor (TNF) production, which is associated with a major apoptotic pathway [32]. Cellphone radiation increases the embryonic mortality of chickens [33].

In vitro, one very elegant set of comparative studies correlating results in human lymphocytes with Chinese hamster cells (V79) has suggested that microwave radiation can induce structural damage in mammalian chromosomal DNA. A significantly higher frequency of specific chromosome aberrations, such as dicentric and ring chromosomes, was observed in irradiated V79 cells than in control samples. Micronuclei were also present in the irradiated V79 cells [34]. Animal studies have demonstrated the neoplastic transformation of a clonal mouse embryo cell line (C3H/10 T1/2) following exposure to modulated microwaves [35]. In other more recent studies, neural cell apoptosis in NGF-differentiated PC12 rat cells has been induced by microwave exposure via the mitochondria-dependent caspase-3 pathway [36]. It represents one of three cellular apoptotic pathways including the extrinsic death receptor-dependent pathway, the intrinsic mitochondria-dependent pathway, and the intrinsic endoplasmic reticulum(ER) stress-mediated pathway [37].

4. Cell studies

Microwave irradiation can produce genotoxic effects in human cells [38]. Induction of micronuclei in human lymphocytes with wide interindividual variability after exposure in vitro to 1800 MHz [39] has been observed and is correlated with specific chromosomal aberrations (acentric fragments and dicentric chromosomes) [40]. Exposure of human peripheral blood lymphocytes to EMR associated with cell phones (830 MHz) leads to chromosomal instability, specifically aneuploidy, which is known to increase cancer risk [41]. Aneuploidy among other kinds of DNA

damage can result in p53-mediated postmitotic apoptosis in human cells [42]. EMR from cell base stations has also been reported to increase the frequency of DNA strand breaks in the lymphocytes of cellphone users and in individuals residing near cell base stations [43, 44].

Cellphone radiation can increase c-fos proto-oncogene activity by more than 40% in embryonic mouse cells and alter c-jun proto-oncogene activity in rat cells [45, 46]. However, there is some conflicting data on this subject, and reports can be inconsistent, while data in humans appear to be lacking. Additionally, there is no evidence to suggest that microwaves can cause point mutations, which are associated with oncogene activation in humans and other mammals [47]. Nevertheless, the c-fos protein can induce cellular apoptosis, and the c-jun gene product has been found to be necessary for neuronal apoptosis in human and other mammalian cells [48, 49]. Microwaves can affect chromatin conformation and histone phosphorylation in human lymphocytes, as well, which may be associated with epigenetic mechanisms at the cellular level [50]. A significant increase in the efflux of calcium ions has been observed in human neuroblastoma cells at extremely low levels of microwave radiation indicating a high degree of sensitivity [51]. This cellular calcium imbalance may reflect the release of calcium ions from internal organelles like mitochondria and the endoplasmic reticulum [ER] as occurs in response to certain heavy metals, and this process is linked to an apoptotic pathway [11].

Microwaves have been reported to induce ERK-mediated apoptosis and cell cycle arrest in a dose-dependent manner in a human natural killer cell line (NK-92) just 1 hour after exposure, which could lead to general immune suppression and the development of tumors [13]. Activation of the Ras/Raf/ERK pathway has been associated with both the intrinsic mitochondrial and the extrinsic death receptor apoptotic pathways [52]. Continuous microwave irradiation (2.45 GHz) can cause Fas-induced apoptosis via the extrinsic death receptor pathway in a human Jurkat T-cell line [14]. Fas is a member of the tumor necrosis factor receptor (TNFR)/ nerve growth receptor (NGR) family. In another recent study, microwave radiation exposure from a GSM cellphone simulator (900 MHz) also resulted in a significant increase in the apoptotic rate of a human T-cell line (Jurkat cells) [53]. In addition, the formation of ROS in normal human peripheral blood mononuclear cells can stimulate apoptosis in response to 900 MHz cellphone radiation. In this case, apoptosis is induced via the mitochondrial pathway and is mediated by ROS [54]. Finally, apoptosis can be stimulated in human brain glioblastoma cells directly in response to microwaves. EMR exposure in the cell base station frequency range [1800 MHz] induces apoptosis-related events such as ROS bursts and oxidative DNA damage, which in turn promote p53-dependent caspase-3 activation through release of cytochrome c from mitochondria [12].

5. A possible mechanism of carcinogenesis

Cellphone radiation can alter c-fos and c-jun proto-oncogene activity, and both these gene products have been implicated in the activation of cell death signal transduction pathways [48, 49].

DNA damage including micronucleus formation, chromosomal aberrations, and DNA strand breaks has been reported in human cells in response to microwave radiation. Certain kinds of

DNA damage like aneuploidy can result in cell cycle arrest and activation of apoptosis. Double-strand breaks in DNA caused by radiation can also signal apoptosis.

The generation of reactive oxygen species in response to microwave radiation has been observed in various studies. Certain carcinogens like UV rays exert some of their carcinogenic effects via the generation of reactive oxygen species in the cell [55]. This is true of X-rays, as well [56]. Certain oncogenic proteins such as Ras also produce elevations in ROS upon stimulation. Many genes and proteins that respond to conditions of oxidative stress within the cell subsequently trigger apoptosis. Because mitochondria are important regulators of cellular redox status, the induction of oxidative stress exhibits its effects upon these organelles to trigger the intrinsic apoptotic pathway via cytochrome c release and caspase cascade activation [57, 58].

Moreover, an increase in the efflux of calcium ions has been observed in human neuroblastoma cells at extremely low levels of microwave radiation, and this cellular calcium imbalance may reflect the release of calcium ions from internal organelles. In this regard, lead perturbs and alters the release of intracellular calcium stores from organelles like the endoplasmic reticulum (ER) and mitochondria [59, 60]. Mitochondria can accumulate large amounts of calcium, for example, in the presence of inorganic phosphate. The rise in calcium results in an upregulation of energy metabolism and an increase in mitochondrial membrane potential. Then, the release of this accumulated calcium through a special channel, permeability transition pore (PTP), can cause mitochondrial depolarization. According to the model of glutamate toxicity, mitochondrial calcium accumulation and resultant membrane depolarization are clearly linked to the initiation of a cell death pathway in mitochondria [61, 62].

Microwaves can also affect chromatin conformation and histone phosphorylation in human lymphocytes. Interestingly, in addition to causing genetic damage via oxidative and non-oxidative mechanisms (DNA adducts), certain carcinogenic heavy metals can cause significant epigenetic changes in cells such as DNA methylation and histone modifications. These can result in gene silencing or reactivation of gene expression [63]. MicroRNAs (miRNAs) are highly conserved, noncoding small RNAs regulating the expression of broad gene networks at the posttranscriptional level and may represent another epigenetic control mechanism. In many cases, the specific effects of such epigenetic changes still appear to be unknown and could conceivably impact major cellular functions like cell death and/or proliferation [64].

Apoptosis is involved in maintaining cell number in tissues, and, although increased cell proliferation is necessary, it is not sufficient for cell transformation to take place. Normally, in multicellular organisms, a dynamic balance exists between cell birth and cell death to retain constant cell numbers throughout adult life. This homeostasis depends on an integrated balance between apoptosis (cell death) and mitosis (cell division) such that these two activities are counterbalanced and equivalent. In fact, this homeostatic balance may contribute a critical defense mechanism of the cell to various genotoxic agents such as carcinogens [65].

A permanent loss in homeostatic equilibrium between cell division and cell death may be a critical determinant in the transition to tumorigenesis. The increased proliferation in preneo-plastic lesions is often accompanied by a parallel increase in cell death, at least in the initial stages of transformation to cancer. Quantitative histological studies in the rat liver model

have revealed that the rate of apoptosis tends to increase from normal to preneoplastic to malignant cells [66]. Comparative studies with the rat bladder have also suggested that apoptosis is closely linked to chemically induced carcinogenesis [67]. Additional support for this transition comes from a variety of other models [7]. However, ultimately, tumor formation only seems to occur once the cancer cells have become resistant to apoptosis while continuing to proliferate. In fact, acquired resistance to apoptosis appears to be a pivotal event in cell immortalization and the transition to malignancy [65].

In summary, various laboratory studies on animals and certain human data [68] are suggestive that tumor formation requires at least two discrete events to take place in response to a carcinogen. The first involves an elevation of apoptosis in a particular tissue due to a genetic predisposition, stress, or mutation. The second confers resistance to apoptosis in that same tissue resulting in the formation of an abnormal growth due to a dysregulation of cell number homeostasis. Moreover, there is some evidence to suggest that both these events can be reversible when treated with a selective apoptotic agent and, hence, they may be either genetic or epigenetic in nature.

Thus, according to this new model, apoptosis becomes an important focus of study and key determinant of carcinogenic potential for any particular chemical or other complete carcinogen being studied, especially in normal, non-transformed cells derived from the target tissue [11].

In the microwave radiation exposure model, there are a number of cellular processes and responses that appear to lead to the endpoint of an increased rate of apoptosis in both animals and humans. These parameters include DNA damage, alterations in gene expression, metabolic perturbations in intracellular calcium levels, effects on the immune system involving decreases in natural killer cells and T lymphocytes, and bursts in ROS activity. All these biochemical effects represent early events that can trigger or are linked to apoptosis and, therefore, could be involved in initiating an apoptotic model of carcinogenesis as described above.

6. Discussion

Briefly, epidemiological data on the human effects of microwave radiation suggest a predom-inance of brain tumors and leukemia. In vivo and in vitro animal studies point to genotoxic effects that can trigger apoptosis and detrimental effects on the immune system. Human cell studies corroborate the genotoxic effects of microwave radiation and its ability to cause vari-ous kinds of DNA damage resulting in cell death. Possible immune effects are also recorded. These results are in keeping with a two-stage apoptotic model of carcinogenesis [11].

The induction of apoptosis by microwaves in human and rat neural cells and in human lymphocytes correlates well with the increased incidence of brain tumors and leukemia epide-miologically associated with the high-frequency radio waves emitted by cellphone towers. However, further studies need to be conducted on the apoptotic potential of microwaves in non-transformed neural and human lymphocytes at 1800–1900 MHz in order to test this

parameter definitively since significant biochemical differences can exist between transformed and non-transformed cells. Blood cells of children should also specifically be tested since they are susceptible to leukemia from high power voltage (HPV) lines, which emit low-frequency radio waves. The developing tissues of children have already been found to be more susceptible to the penetration of cellphone radiation. According to a new approach to cancer risk assessment, if apoptosis is induced in these normal tissues from adults and children, along with the epidemiological data, this would be sufficient criteria to establish cellphone tower EMR as a complete carcinogen providing that microwave exposure is at a high enough specific absorption rate (SAR).

As an example, sufficiently high SAR levels for microwave radiation are likely to be achieved only very close to or directly in front of cellular antennas mounted on a roof, whereas a distance of up to 400 meters from cellphone towers, which emit more EMR, has been found to be associated with an increased cancer incidence. In any case, access to such rooftop areas with cellular antennas should be restricted or limited [69]. Actually, defraying the total EMR load in this way may be one potential method of decreasing total human exposure in urban neighborhoods. Dividing up the EMR load between several buildings in an urban area could help to minimize overall individual microwave exposure, while having one large cellphone tower in the same area would tend to maximize the microwave exposure of a few.

According to various animal studies, there appears to be a significant effect of microwaves in the cellphone tower frequency range on mammals, avian species, and insect pollinators such as honey bees. There also appears to be a negative impact on plant life in the vicinity of cellphone towers. Decreases in fruit and other crop yields could translate into economic losses. As a result, some countries like India have already taken positive action against the potential threat of cellphone tower EMR to wildlife by proposing to have EMR levels audited and recognized as a pollutant and passing a special law to safeguard the surrounding environment. Other countries should also follow suit in setting safe environmental limits on EMR emission levels from cellphone towers in order to preserve the urban flora and fauna. Such safety standards should always be based on the latest research and must be subject to constant revision as new data become available.

Author details

Chanda Siddoo-Atwal[1,2,3,4]

Address all correspondence to: moondustcosmetics@gmail.com

1 University College London, UK

2 Simon Fraser University, Canada

3 Medical College Wisconsin, Milwaukee, WI, USA

4 President and Primary Biochemist of Moondust Cosmetics Ltd., Canada

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Apoptosis is an essential biochemical process in cell turnover, development, and chemical-induced cell death. Current knowledge and ongoing research of apoptosis highlight our understanding in designing the therapeutic approaches for several diseases. This book covers four main sections: "Apoptosis and Necrosis," "Apoptosis Inducers," "Proteasome and Signaling Pathways in Apoptosis," and "Radiation-Based Apoptosis." The first section implicitly describes the differences between apoptosis and necrosis processes. The following section elaborates the small molecule-induced apoptosis. Then, the third section deals with proteasome and signaling pathways and finally, resistance to chemotherapy and electromagnetic radiation is covered in the last section. Overall, the book deals with pathways for manipulating apoptosis and provides a unique perspective to the scientists.

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