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Treatment of Metastatic Melanoma

Edited by Rachael Morton



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Meet the editor



Dr. Rachael Morton is a research fellow in the School of Public Health, Sydney Medical School at the University of Sydney, Australia. She has post-graduate qualifications in clinical epidemiology, and health economics and was the clinical trials manager for the Melanoma Institute Australia (formerly Sydney Melanoma Unit) from 2004-2007. Rachael is an executive member of the Australia and New Zealand Melanoma Trials Group (ANZMTG) and board member of the Melanoma Network. She has published on the role of sentinel node biopsy in melanoma treatment, the reproducibility of lymphoscintigraphy, and the value of diagnostic imaging in melanoma follow-up. She is a chief investigator for a randomized controlled trial into high dose vitamin D for patients at risk of melanoma recurrence, and associate investigator for a trial of whole brain radiotherapy following surgical resection of melanoma brain metastases. Rachael's current melanoma research includes the evaluation of post treatment follow-up of stage I/II cutaneous melanoma from the perspective of clinicians and patients.

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Preface

The National Cancer Institute in the United States estimates that more than 70,000 new cases of melanoma will be diagnosed in the US in 2011 with the disease causing approximately 8,790 deaths. The highest incidence of cutaneous melanoma is seen in Australia and New Zealand where it represents 9.5% of all cancers. Here the risk of being diagnosed by age 85 is 1 in 15 for men and 1 in 24 for women. The risk of melanoma increases with exposure to ultra-violet radiation, increased numbers of dysplastic naevi, immunosuppression, a history of melanoma in a first degree relative, fair skin with light eye or hair colour, and a previous history of melanoma or non-melanoma skin cancer.

The classification of cutaneous melanoma is according to the American Joint Cancer Committee (AJCC)/UICC melanoma staging system (2009). The diagnosis of melanoma is confirmed by surgical excision and histological examination of the biopsy specimen, with superficial spreading melanoma the most common histological type. The staging of melanoma is based on tumour characteristics that include Breslow thickness, Clark level of invasion, tumour mitotic rate and ulceration (stage I/II); the involvement of lymph nodes (stage III); and the involvement of distant sites such as skin or other organs (stage IV). While the five-year survival for patients with stage I or II melanoma ranges from 53-97% depending on sub-stage, patients diagnosed with stage IV disease have a considerably worse outcome.

In disease localized to the primary tumor site and/or lymph nodes, surgery remains the mainstay of treatment of this malignancy. Many improvements in surgical techniques have occurred in the last decade, including sentinel node biopsy for the staging of patients with of cutaneous melanoma of intermediate thickness. Adjuvant radiotherapy to regional lymph node fields is often recommended after surgery for patients at high risk of relapse. Isolated limb perfusion, electrochemotherapy, and photodynamic therapy continue to be evaluated for treatment of stage IV disease. However, the greatest excitement in new treatment has been with the targeted therapies for genetic mutations. In particular, there have been promising results with partial and complete tumor responses in stage IV disease from early phase trials of the B-RAF kinase inhibitors.

This book provides an insight into the current therapeutic treatment options for patients with metastatic melanoma and is relevant to clinicians and researchers

worldwide. In addition, an update on current clinical trials for melanoma treatment has been included, and two chapters have been included in which the treatment of oral and uveal melanoma is discussed.

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Part 1

Treatment Options for Cutaneous Melanoma

Cutaneous Metastases from Malignant Melanoma: Clinical Features and New Therapeutic Perspectives

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1. Introduction

In this chapter, cutaneous metastases from malignant melanoma will be analyzed from a clinical and a prognostic point of view.

This non rare condition is often distressing for the patient, as cutaneous lesions increase progressively in number and size and are frequently worsened by ulceration, bleeding and pain.

After a general introduction about the incidence of cutaneous involvement in melanoma natural history, clinical classification of skin metastases will be provided. Then, the impact of cutaneous localizations on prognosis will be evaluated. In the last paragraph, the different therapeutic options for the management of patients with loco-regional or diffused cutaneous metastases will be reviewed.

2. Epidemiology

Skin metastases from solid tumor are not rare. They affect an estimated percentage of patients ranging from 0.7 to 9% in several literature series (Spencer & Helm 1987, Lookingbill, Spangler & Helm 1993, Schwartz 1995, Hu et al. 2008), in the late phases of disease progression or, in more than 7% of patients, as first sign of disseminated disease (Lookingbill, Spangler & Helm 1993, Rosen 1980). Breast cancer is the most commonly involved tumor, accounting for more than 60% of cases of cutaneous spread, followed by colon carcinoma (Krathen, Orengo & Rosen 2003). Moreover, cutaneous metastatic disease is commonly seen with cancer of the lung, kidney and ovary and with sarcoma, lymphoma or leukemia.

As expected, tumor types are differently distributed among the two genders: lung, colon and head and neck tumours together with melanoma account for the majority of skin metastases in males; whereas, breast cancer is the most common neoplasm related to the development of cutaneous secondary lesions in females (Hu et al 2008).

Focusing on melanoma, skin metastases represent a relatively frequent event in the natural history of the disease and can develop in early as well as in late stage of disease. Cutaneous or subcutaneous lesions arise in 10-17% of patients affected by melanoma and almost the 50% of patients with metastatic disease develops skin involvement (Lookingbill, Spangler & Helm 1993, Schwartz 1995, Krathen, Orengo & Rosen 2003).

No specific clinical or histological characteristics were found in patients with cutaneous metastases from melanoma if compared to those with visceral localizations (Savoia et al 2009). However, known risk factors related to prognosis impact on the metastatic melanoma potential.

3. Classification

On the basis of the distance from the primary melanoma, skin metastases are described as local recurrences, in transit disease or distant metastases.

True local recurrences are defined as the reappearance of melanoma in -or contiguous with- an excision scar or a graft and bearing an in situ component (Olsen et al 1970; Brown & Zitelli 1995). The prognosis of local recurrence defined strictly in this way is much better than that associated with in transit disease and 5-year survival rate is related only to the thickness of the primary melanoma. These recurrences are in fact considered as a result of a uncompleted resection of primary melanoma, and are for this reason becoming rare.

In-transit disease (satellitosis) indicates cutaneous or subcutaneous disease between the primary site and the regional lymph nodes. Satellite nodules and in-transit disease are associated with worse prognosis (super imposible to a melanoma with nodal metastases; stage III disease), and the distance of cutaneous deposits from the primary site has no prognostic significance (Balch et al 2009).

Distant cutaneous metastases are defined as tumour lesions that grow in any skin site over the regional lymph nodes. The presence of any distant metastases delineates a stage IV disease, even if patients with sole distant skin metastases (and normal serum LDH levels) have a relatively better prognosis if compared with those of other metastatic patients (Balch et al 2009). Obviously, distant skin melanoma localization can appear together with or in absence of other visceral metastases. Stage and prognosis vary according to AJCC classification as shown in table 1 (Balch et al 2009).

Clinical Staging				Pathological Staging			
0	Tis	N0	M0	0	Tis	N0	M0
IA	T1a	N0	M0	IA	T1a	N0	M0
IB	T1b	N0	M0	IB	T1b	N0	M0
	T2a				T2a		
IIA	T2b	N0	M0	IIA	T2b	N0	M0
	T3a				T3a		
IIB	T3b	N0	M0	IIB	T3b	N0	M0
	T4a				T4a		
IIC	T4b	N0	M0	IIC	T4b	N0	M0
III	any T	N 1-3	M0	IIIA	T1-T4a	N1a/2a	M0
				IIIB	T1-T4b	N1a/2a	M0
					T1-T4a	N1b/2b	M0
					T1-T4a/b	N2c	M0
IIIC	T1-T4b	N1b/2b/2c	M0				
	any T				any T	N3	M0
IV	any T	any N	M 1	IV	any T	any N	M 1

Table 1. Clinical and pathological staging, AJCC 2009.

4. Clinical features

Skin metastases from melanoma can arise as single or multiple nodules. The most common presentations of cutaneous metastatic disease are brown to black or skin colored papules and nodules, sometimes ulcerated. In the majority of these cases cutaneous metastases were correctly identified by the clinician before the pathologic diagnosis was given; dermoscopy could help in diagnosis, even if skin melanoma metastases have often aspects that are indistinguishable from the characteristic pattern of blue nevi (Carlos-Ortega, de Oca-Monroy & Isyta-Morales 2008). Epidermotropic melanoma metastases are histopathologically characterized by aggregates of atypical melanocytes within the dermis with thinning of the epidermis. Usually there is no lateral extension of atypical melanocytes within the epidermis beyond the concentration of the metastases on the dermis. Metastases differs from primary melanoma by the absence of inflammatory infiltrate and junctional activity, even if a prominent lymphocytic infiltrate can be sometimes observed. In few cases metastatic cells are small and nevoid, with few or any mitoses and differentiation from compound nevi is difficult (Elder E et al, 2005. Tumours and Cysts in dermis and Subcutis, in: *Lever's histopathology of the skin*. Lippincott Williams&Wilkins, Philadelphia).

Less frequently, a wide morphological spectrum of lesions has been described, including erythematous patches or plaques, inflammatory erysipela-like lesions, diffuse sclerodermiform lesions with indurations of the skin ("en cuirasse" metastatic carcinoma), telangiectatic papulovesicles, purpuric plaques mimicking vasculitis, and alopecia areate-like scalp lesions (Saeed, Keehn & Morgan 2004, Sarya et al 2007). In these cases, clinical diagnosis could be more challenging and metastases can be suspicious for benign entities (Figure 1).



Fig. 1. Clinical features of local recurrences that are defined as the reappearance of melanoma in or contiguous with an excision scar or graft and bearing an *in situ* component.

Moreover, there are also rare cases of so-called zosteriform metastases, with vesicobullous herpetiform lesions or papules and nodules distributed along one or more dermatomes. A previous Varicella Zoster Virus (VZV) infection or widespread lymphatic obstruction by tumor cells can justify the zosteriform pattern (Figure 2).



Fig. 2. Zosteriform metastases among thoracic dermatomes.

However, zosteriform metastases, as well as the rare skin metastases occurring on skin graft donor site, could be explained as a Koebner phenomenon (Savoia et al 2009, Marengo et al 2009).

From a clinical point of view, if bleeding and super-infection are not present, superficial skin metastases are usually asymptomatic, even if patients frequently report localized pain and paresthesiae anticipating the onset of clinically evident cutaneous lesions; these symptoms are related to oedema and mechanical stress on the near tissues and usually disappear in a few days. On the other hand, when subcutaneous lesions grow deep infiltrating muscles or nerves become very painful. The management of pain in these cases could be difficult and requires a multidisciplinary approach.

As mentioned earlier, bleeding and super-infections are the most frequent complications of skin metastases and can significantly impact on the patient's quality of life (Kaheler, Egebarts & Hauschild 2010). These complications can also compromise general conditions. Massive bleeding from cutaneous metastases could become life threatening; sepsis related to the bacterial dissemination of infected metastases represents an uncommon but not rare event, that lead to septic shock and death (Figure 3).



Fig. 3. Infected diffuse metastases from malignant melanoma

5. Pattern of cutaneous localizations

Cutaneous secondary lesions can occur on all anatomic sites, with skin metastases from other solid tumours more frequently found on the head, neck, anterior chest and abdomen, whereas lower extremities are rarely involved (Schwartz, 1995). Conversely, skin metastases from melanoma are more frequently observed on the back in men and on the lower limbs in women. These different patterns of cutaneous localizations among sex can be explained by the fact that in more than 30% of cases, secondary cutaneous localizations occur in the same anatomic area of the primary (Savoia et al 2009).

6. Clinical course and prognosis

In more than half of the cases, skin represents the first site of metastatic involvement after the primary melanoma diagnosis. In about one third of cases, patients develop skin involvement after evidences of regional lymph nodal metastatic disease.

The finding of concomitant distant cutaneous, visceral and nodal metastases account for more than 10% of cases, whereas skin involvement after visceral dissemination is rare, and occurs only in about the 3% of patients (Savoia et al 2009).

Cutaneous metastases are loco regional in nearly 80% of cases, whereas distant metastases were documented in the remaining 20% of patients. A different pattern of cutaneous metastases was related to the time of onset: when cutaneous metastases arise as the first site of relapse, there is a significant higher percentage of locoregional localizations, whereas distant skin involvement was more frequently observed after visceral involvement. No significant differences were found between patients with regional and those with distant metastases regarding to the known risk factors, such as Breslow thickness, Clark level, histotype of the primary melanoma and ulceration (Savoia et al 2009).

It is noteworthy that in patients with distant metastases, primary melanomas arose predominantly at trunk and back, whereas patients with cutaneous loco-regional spreading were affected mainly by primary located at leg and foot. As we know, loco-regional metastases develop as a result of tumour cell embolization in the dermal lymphatic vessels between the primary tumour site and the draining regional lymph node basin; lymphatic stasis to lower limbs consequent to nodal dissection represents an additional risk factor for cutaneous locoregional dissemination.

In contrast, the correlation between disseminated skin lesions and primary melanoma located to the trunk could be explained by the fact that the lymph drainage of this region is

not strictly dependent on a single station, but it could be resulted from more than one lymphatic basin, together with a possible role of haematogenous spreading.

Disease free survival evaluated from the first melanoma diagnosis varies in relation to the first site of metastatization. In our experience, loco regional cutaneous relapses develop early, but show a very late progression to visceral disease. On the contrary, patients with disseminated skin lesions as first site of relapse had a longer disease free interval from the first diagnosis but a shorter time to progression to visceral metastases (Savoia et al 2009).

7. Treatment and clinical management

The choice of the modality of treatment for cutaneous melanoma metastases depends on several factors, including location and number of lesions, presence of systemic involvement, age and general health conditions of patients. Moreover, the prognostic differences between patients with loco-regional and distant skin metastases justify different approaches in their clinical management.

Important therapeutic options including surgery, isolated limb perfusion, local or systemic chemo- and immuno-therapy and radiotherapy are discussed in detail below.

7.1 Surgery

Surgery is the gold standard and represents the most effective treatment for limited in-transit disease, when technically feasible. It is an adequate treatment when the lesions are relatively small and clustered in a reasonable circumscribed area. Primary melanoma should be excised widely with a 1-2 cm margin depending on Breslow thickness, whereas wide surgical margins are unnecessary for the treatment of cutaneous metastases. Usually, metastases are clearly demarcated from the surrounding normal dermis and overlying epidermis and the better approach is the complete macroscopical excision of the lesion. When microscopical involvement of margins is documented, reintervention is not mandatory (Hoekstra, 2008).

If technically possible, direct wound closure is to prefer; the second choice is represented by skin graft, because plastic surgical reconstruction can affect the lymphatic drainage pattern. Palliative treatment should be considered when results in the control of local complications (e.g. bleeding) and/or in a consistent quality of life improvement.

Amputation should be only considered as palliation for imminent exsanguinating haemorrhage or fungation unacceptable for the patient.

7.2 Isolated limb perfusion

Isolated limb perfusion (ILP) -firstly described by Creech and Kremenz in 1958- can deliver high doses of cytotoxic agents to a limb, minimizing systemic toxicity. The dose received regionally can be up to tenfold higher than the systemic mean tolerated dose. Isolated limb perfusion is widely indicated for patients with advanced or recurrent in-transit disease, showing a complete response rate around the 50% in the majority of the published series, with an overall response rate up to 80% (Lens & Dawes 2003, Rossi et al 2010). On the contrary, the role of isolated limb perfusion as adjuvant therapy is still debated (Hoekstra 2008). The tumour response after perfusion is the only demonstrated prognostic factor affecting local control of the disease and overall survival (Rossi et al 2010).

The usual agent employed is melphalan, with or without tumour necrosis factor (TNF); TNF increases response rate thanks to its selective disruption of the tumour microvasculature, with

a consequent ischemic damage of melanoma cells, even if seems not to influence the long term local control (Di Filippo et al 2006). Dacarbazine is less effective when administered regionally; other combinations of cytostatics (dactinomycin, nitrogen mustard, vindesine, thio-TEPA) have also been proposed but the published series are too small to give absolute conclusions (Daryanani et al 2000, de Wilt et al 2000, Hoekstra 2008). Hyperthermia, with temperature between 39° to 41° act synergically with high dose chemotherapy, even if can exacerbate loco regional toxicity (Hoekstra 2008).

General anesthesia is required. However, age does not represent a contraindication to ILP. Systemic side effects, due to drug releasing into the systemic circulation are rare and mainly represented by nausea, vomiting and mild bone marrow suppression. Local toxic reactions are more frequently described and ranges from mild erythema to deep tissue inflammation; nearly 25% of patients develop neuropathy or pain, whereas chronic edema is usually related to lymphadenectomy (Bonifati et al 2000, Rossi et al 2002).

7.3 Electrochemotherapy

Recently, electrochemotherapy (ECT) has been proposed as a new treatment modality for skin metastases of different malignancy, including melanoma. ECT enhances membrane permeability by electric pulses thus permitting a major drug delivery in neoplastic cells and a better cytotoxic effect.

Bleomycin and cisplatin are the drugs more frequently used in ECT with an increased efficacy up to 8.000-fold for bleomycin, and up to 80-fold for cisplatin (Gaudy et al 2006).

The ECT technique requires only a regional anesthesia or mild general sedation with a lower duration if compared to isolated limb perfusion. With respect to ILP, ECT shows a minimal systemic toxicity; treatment is generally well tolerated; side effects were mainly represented by erythema and edema at the site of treated lesions, superficial erosions, scars and permanent marks from the electrodes (Quaglino et al 2008). Thus ECT can be performed also in patients with major co morbidities.

The first large study about effectiveness of ECT in melanoma treatment, was the multi center European Standard Operating Procedure of Eelectrochemotherapy (ESOPE), based on the new Cliniporator™ Electric Pulse Generator; this study enrolled twenty melanoma patients, with an overall response rate of more than 20% (Marty et al 2008). Several papers recently published confirm these encouraging results of ECT in the control of skin metastases (Campana et al 2009, Moller et al 2009). In our experience, the global response rate was of 79.4, with a percentage of complete remissions of 23.2%(Quaglino et al 2008); complete response was defined in accordance World Health Organization (WHO) guidelines as the total clinical disappearance of the tumor (WHO. From *Handbook for Reporting Results of Cancer Treatment*, vol 48; pp 22-27. Geneva, 1997).

The lesion size was the most predictive parameter for response; response rate for larger lesions was significantly lower. Moreover, a second limit is represented by the possible relapse of new lesions on untreated areas: ECT represent in fact a local treatment. However, it is possible to repeat ECT, both on new metastases in untreated areas and on already treated lesions with a previous partial remission or no changes. In our experience, new responses were obtained in about 60% of retreated lesions.

Appropriate dressing should be performed with the aim to control ulceration of cutaneous tumours, local infectious complications and to ensure an acceptable quality of life.

7.4 Radiotherapy

The effectiveness of radiotherapy in the treatment of melanoma metastases is still debated. A poor response was historically observed on *in vitro* cultures from melanoma cells treated with external-beam radiation (Barranco, Romsdahl & Humphrey 1971). So, radiotherapy was mainly used as palliation when disease was too extensive for surgery and other modalities of treatment were inadvisable or ineffective in stage III and IV melanoma patients. However, a retrospective review (Fenig et al 2009) showed a 52% response rate in stage IV patients who received radiotherapy with palliative intent and others studies (Sause et al 1991; Seegenschmeid et al 1999) demonstrated an overall response rate ranging from 60 to 79% for stage III disease. Moreover, disease-free and overall survival seems to be significant longer in patients who received radiotherapy (Olivier et al, 2007).

7.5 Chemotherapy

Usually, chemotherapy plays a role in the treatment of stage IV melanoma patients with visceral metastases. Regarding cutaneous metastases, chemotherapy can be used in patients with wide spread skin lesions not eligible for local treatments, with or without a concomitant visceral involvement.

Chemotherapy could be used also in stage III, when other loco-regional treatments have failed or are technically not feasible (e.g. cutaneous lesions diffused at trunk or back).

The global response rate is less than 25% for single agent treatment. The gold standard is still represented by Dacarbazine; new molecules as Temozolomide and Fotemustine showed a super imposable disease-free and overall survival, with major toxicity (Middelton et al, 2000). Combination regimens or chemo-immunotherapy give higher response rate but also more severe side effects. However, response to chemo-immunotherapy is not related to statistically significant benefit in term of overall survival when compared with a single-agent treatment.

Recently, Ipilimumab, a human anti-CTLA4 monoclonal antibody showed objective responses or disease stabilization in patients with advanced melanoma (O'Day et al, 2010). No data about the effectiveness of Ipilimumab in the treatment of cutaneous metastases are available, even if several studies are ongoing.

The response rate for *in transit* metastases treated with dacarbazine is 15-20%; the majority of the responses are partial with a median response duration less than 6 months. Chemoimmunotherapy showed also no survival benefit (Hoekstra 2008).

Finally, the clinical efficacy of RAF inhibitors in BRAF mutated melanoma patients are under evaluation after some encouraging preliminary report; a phase I study on patients with both disseminated cutaneous and visceral metastases reported an 81% of clinical responses in patients treated with selective BRAF inhibitor, with a median time to progression of 6-9 months (Flaherty, 2010).

7.6 Others

7.6.1 Laser ablation

Patients with small (<2 cm) and superficial lesions who are not suitable for isolated limb perfusion, ECT or other conventional modalities of treatment can be considered for carbon dioxide (CO₂) laser ablation. This therapy is minimally invasive: only local anesthesia is required and the resultant defect does not require surgical closure but can be covered with a dressing until secondary healing (Lingam & McKay, 1996; Gibson, Byrne & Mc Kay, 2004).

So, can be considered a minimally invasive and effective method of palliation; the role as first line treatment is still debated, in fact the technique can be used to treat only visible and superficial lesions, while deep subcutaneous metastases, large volume lesions and microscopic disease can not be treated with laser ablation (Gimbel, Delman & Zager, 2008).

7.6.2 Cryosurgery

This technique uses temperatures from -50°C to -60°C (with nitrogen spray) to obtain direct tissue destruction. The heat transfer results in vascular stasis, ice crystal and disruption of cell membranes, pH changes, hypertonic damage and thermal shock that lead to tissue damage and necrosis. Low temperature causes the development of a bulla and a secondary healing with a scar. After the introduction of laser ablation and ECT, cryosurgery is less frequently used in the treatment of cutaneous metastases from melanoma (Hoekstra, 2008).

7.6.3 Intralesional therapy

The intralesional injection of bacillus Calmette-Guérin (BCG) was the first immunotherapy used in the local treatment of cutaneous metastases from melanoma. This procedure was accompanied by severe complications such as ulceration, skin necrosis, and superinfection without significant improvement of lesions treated (Tan & Ho, 1993). Other drugs such as IL-2, INF alpha and dinitrochlorobenzene, with or without systemic dacarbazine, were more recently used as intralesional therapy (Radny et al, 2003; Strobbe et al, 1997); results are still debating and further investigations are necessary. Encouraging results regarding high-dose intratumoral IL-2 administration in melanoma patients with cutaneous secondaries has been reported (Weide et al, 2010): a complete local response was described in more than 60% of melanoma patients and seemed to be associated with an increased responses to subsequent chemotherapies. Recently, phase I studies confirmed the safety and an enhanced immune response for intralesional injection of Allovectin in metastatic melanoma patients. Unfortunately, phase II studies showed a complete response in only the 3.1% of treated patients, with an overall response rate of 11.8% and a median time-to-progression of 1.6 months (Beidikan et al, 2010).

Rose bengal has been proposed as a possible intralesional treatment, with an objective response rate ranging from 27 to 69%, related to the dose of injection (Thompson, Hersey & Wachter, 2008). Systemic toxicity is low, even if phototoxic reactions have been described.

An interesting therapeutic option is represented by topical imiquimod. It enhances the immune system activity leading to an induction of melanoma-specific cytotoxic T-cells by cross presentation of melanoma antigen by dendritic cells. Partial remission of locoregional cutaneous metastases treated with imiquimod was demonstrated (Wolf, Richtig, Kopera & Kerl H, 2004).

8. Conclusions

Skin metastases from melanoma are a frequent finding in the natural history of the disease with various clinical, morphological and histopathologic backgrounds. The presence of progressively increasing metastases is often distressing for the patient, and ulceration, bleeding and super-infections can negatively impact on the life-quality. To date, many treatments are available for the clinical management of these lesions. Thus, clinicians should be informed about the prognostic implications and the therapeutic options in order to choose the best cost-effectiveness treatment modality.

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Part 2

Surgical Treatment of Melanoma

Impact of Sentinel Node Biopsy on Outcome in Melanoma

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1. Introduction

The worldwide incidence of malignant melanoma is increasing at an alarming rate. The importance of diagnosing nodal metastatic disease has impacted significantly on the accurate staging and stratification of melanoma patients. As a minimally invasive procedure with low morbidity, sentinel lymph node mapping allows for a detailed histopathologic evaluation involving multiple sections, H&E staining in combination with immunohistochemical staining of the node with the highest chance of containing metastatic foci. Controversy exists regarding the appropriate selection of patients for sentinel lymph node biopsy, particularly among patients with thin melanomas (< 1 mm Breslow thickness), thick melanomas (> 4 mm Breslow thickness), locally recurrent melanoma, nodular melanomas and those affecting the head and neck region. Furthermore, debate continues with regard to false-negative rates, managing in-transit disease, therapeutic benefit and alternatives, such as ultrasound guided biopsy.

In malignant melanoma, no standard systemic adjuvant therapy with confirmed impact on overall survival has been identified thus far for clinically node negative stage I-II patients after excision of the primary, or for clinically node positive stage III patients after lymph node dissection for metastatic regional node involvement. Thus some argue about the initial merits of performing the sentinel node procedure at all.

The aims of this book chapter are

1. to examine the impact of sentinel node biopsy on outcome in melanoma,
2. determine the effect, if any, of stage migration in melanoma
3. to clarify the impact of the different clinical sites on outcome,
4. to ascertain the reasons behind a lack of universal adoption of sentinel node biopsy in melanoma and
5. to critically assess other emerging strategies in the management of melanoma including frozen section analysis of the sentinel node, imprint cytology of the sentinel node, targeted assessment of the regional lymph node basin, the use of risk stratification algorithms of histological factors of the primary tumour and microRNAs.

2. Impact of sentinel node biopsy on outcome in melanoma

The key to survival for patients with melanoma is early detection and treatment of metastatic disease as this may improve disease-free and overall survival rates (Pacifico,

Grover, and Sanders 2004). The status of the regional lymph node basin has been widely shown to be the most important prognostic indicator for patients diagnosed with cutaneous melanoma (Balch et al. 2001). The disease-free survival and overall survival is dependent on the initial disease burden, thus melanomas less than 1mm rarely metastasise while at least 25% of melanomas between 1.5 and 4.0mm and greater than 60% of melanomas greater than 4.0mm thick will have lymph node metastases at presentation (Balch et al. 2001). The disease also depends heavily on the stage at presentation. Patients with early stage disease (i.e. < 1mm thick) achieve long-term survival in more than 90% of cases. However, patients with melanomas greater than 1.0mm thickness have survival rates ranging from 50%-90% (Balch et al. 2001).

The introduction of the sentinel lymph node biopsy (SLNB) technique for the evaluation of patients with truncal and extremity melanoma by Morton et al in 1992 showed that the status of the SLN accurately represented the status of the entire nodal basin from which it was obtained. This study highlighted a novel technique of identifying patients with occult nodal metastasis who warranted possible further therapeutic lymphadenectomy and adjuvant therapy, whilst also sparing the remaining 80% of patients without regional disease the morbidity associated with a formal lymphadenectomy procedure (Morton et al. 1992).

The benefits to performing SLNB versus elective lymphadenectomy are well supported (Pawlik, Ross, and Gershenwald 2004). SLNB is associated with less morbidity and is cheaper to perform (Wrightson et al. 2003). Historically two studies showed a survival benefit in patients who had SLNB performed (Dessureault et al. 2001; Kretschmer et al. 2004). Furthermore a positive sentinel node, Breslow thickness, age and male gender were all independent predictors of overall survival on multivariate analysis (Kretschmer et al. 2004).

The proven benefits for performing SLNB at the time of oncological wide local excision of a primary melanoma in any patient, include both prognostic and staging information, the potential therapeutic impact of a completion lymph node dissection in those with a positive SLN and also has implications of SLN status for adjuvant therapy decisions or entry into pertinent clinical trials. Because the status of the regional lymph nodes is the most single important prognostic factor for patients with melanoma, obtaining this information is essential (Shaw et al. 1985).

The preliminary findings of the MSLT-1 trial (Multicentre Selective Lymphadenectomy Trial) represent the first randomised prospective clinical study to show a potential survival advantage to performing sentinel lymph node biopsy in patients with melanoma (Morton et al. 2006). The study included 1269 patients with intermediate thickness (1.2-3.5mm) primary melanomas randomised to either a wide local excision and observation of regional lymph nodes with lymph node dissection if nodal relapse occurred (n=500) or to a wide local excision and SLNB with immediate regional lymphadenectomy if nodal micrometastases were found (n=769).

There was no difference in overall 5-year melanoma-specific survival rate; 87.1% for the SLNB and 86.6% in the control arm. However, the study did show improved disease-free survival in those patients who underwent the SLNB compared to those in the nodal observation arm group; the 5-year survival rate for node positive melanoma was $72.3 \pm 4.6\%$ and $90.2 \pm 1.3\%$ in the node negative group (hazard ratio for death, 2.48; 95% confidence interval, 1.54 to 3.98; $P < 0.001$).

The incidence of sentinel-node micrometastases was 16.0% and the rate of nodal relapse in the observation group was 15.6%. The corresponding mean number of tumour-involved

nodes was 1.4 in the biopsy group and 3.3 in the observation group ($P < 0.001$), indicating disease progression during observation. Among patients with nodal metastases, the 5-year survival rate was higher among those who underwent immediate lymphadenectomy than among those in whom lymphadenectomy was delayed (72.3+/-4.6% vs. 52.4+/-5.9%; hazard ratio for death, 0.51; 95% confidence interval, 0.32 to 0.81; $P = 0.004$) (Morton et al. 2006). The study authors concluded that the staging of intermediate-thickness (1.2 to 3.5 mm) primary melanomas according to the results of SLNB provided important prognostic information. However, the study did not show a clear survival advantage associated with SLNB.

Further subset analysis from MSLT-1 comparing patients with a positive SLNB + immediate lymphadenectomy versus those in the nodal observation group showed that there was significant progression to more advanced nodal disease in the nodal observation group and more importantly that there was a significant survival advantage in those undergoing immediate lymphadenectomy.

In order to appreciate the full potential applicability of the results from MSLT-1, it is important to consider the results from the WHO Truncal Melanoma international multicentre randomised trial which examined the use of early elective lymph node dissection (ELND) at the time of WLE of truncal melanomas $> 1.5\text{mm}$ versus delayed lymphadenectomy until appearance of regional-node metastases (Balch et al. 2000). Firstly, this trial in addition to one carried out in Europe and published 2 years before (Cascinelli et al. 1998) under the title of WHO Melanoma Programme, did show a trend for improved survival in those patients who had ELND at the time of WLE, however this was not significant. But they did show improved 5-year survival rates in patients with occult regional node metastases 48% versus 27% in patients in whom the regional node dissection was delayed until the time of appearance of regional node metastases, which indeed was significant. The patients with regional nodes that became clinically and histologically positive during follow-up had the poorest prognosis. They concluded that SLNB may become a tool to identify patients with occult node metastases, who could then undergo node dissection (Balch et al. 2000).

These same authors showed that nodal micrometastatic deposits detected by the SLNB will become clinically relevant disease eventually therefore the logic of removing these involved lymph node deposits early may improve patient prognosis (Morton, Cochran, and Thompson 2007). Numerous reasons are cited in an article by Ross et al detailing exactly why the microscopic metastases in sentinel lymph nodes would most likely progress to palpable disease if left intact (Ross and Gershenwald 2008).

Most recently, an international panel comprising a cross section of expert melanoma surgeons who have contributed data and leadership to further investigate the role of SLNB in melanoma recently produced a consensus statement, outlining their overall interpretation of current evidence, as a guide to clinical treatment of patients with clinically localized melanoma. They agreed that SLNB is standard of care in current practice because it is incorporated in staging guidelines from the AJCC, incorporated in the treatment guidelines from the National Comprehensive Cancer Network, and practiced by most specialty surgeons who treat melanoma in the United States, Australia and Western Europe (Balch et al. 2009).

3. Stage migration effect

Oncologically, the prognosis for malignant disease is largely determined by the metastatic potential of the primary tumour. Some authors argue that we cannot alter prognosis by

early detection and surgical intervention of involved regional nodes, highlighting the balance between nihilistic pre-determinism and active management. As an oncological community, we have a greater understanding of the mechanisms of action for haematological spread versus lymphatic spread. For many malignancies we know that regional lymphadenectomy improves survival. As to just how far this lymph node dissection is directly therapeutic remains a persistent source of controversy in melanoma.

Many believe that stage-adjusted survival benefit is due in part to the phenomenon of stage migration (*Will Rodgers phenomenon*). In medical stage migration, improved detection of illness (eg when newer technology allows for more sensitive detection of tumour spread) leads to the movement of people from the set of healthy people to the set of unhealthy people. Because these people are not healthy, removing them from the set of healthy people increases the average lifespan of the healthy group. Likewise, the migrated people are healthier than the people already in the unhealthy set, so adding them raises the average lifespan of that group as well. Both lifespans are statistically lengthened, even if early detection of a cancer does not lead to better treatment - because it is detected earlier, more time is lived in the "unhealthy" set of people. It was originally described in 1985 (Feinstein, Sosin, and Wells 1985). Rodgers' original quote - "When the Okies left Oklahoma and moved to California, they raised the average intelligence level in both states" - illustrates the theme. It has been shown that stage migration is responsible at least in part for an apparent improvement in survival for patients with stage III and IV non-small cell lung cancer in the era of PET scanning (Chee et al. 2008). It has also been demonstrated in urological, prostate and laryngeal malignancies (Albertsen et al. 2005; Champion and Piccirillo 2004; Gofrit et al. 2008). A large population-based study assessing the surgical treatment trends for 18449 patients with melanoma in the era of sentinel node biopsy using a SEER database concluded that stage migration is evident with increasing use of SLN biopsy (Cormier et al. 2005).

Furthermore, the theme underlying this phenomenon may no longer be confined to interpreting oncology trials as its use has been cited in other paramedical arenas including healthcare economics. Young et al studied the measured differences in health care utilisation across an indemnity and managed care plan, finding that apparent increases in utilisation in both plans disappeared when viewed together, reflecting the migration of sicker patients from indemnity to managed care plans (Young et al. 1999).

4. Impact of histological site on outcome

While SLNB has a defined role in cutaneous melanomas of the trunk and extremities, several questions remain unanswered with respect to its application in the head and neck region. These account for up 21% of all melanomas diagnosed annually (Gillgren et al. 2000; Golger et al. 2007; Lachiewicz et al. 2008), have worse outcomes relative to melanomas of the trunk and extremities (Gillgren et al. 2000; Lachiewicz et al. 2008), clinically manifest as thicker lesions at their initial diagnosis and thus present at an advanced stage (Gillgren et al. 2000; Hoersch, Leiter, and Garbe 2006). SLNB for melanomas of the head and neck regions is limited by technical difficulties with specific concern surrounding damage to vital structures such as the facial nerve (Eicher et al. 2002). There is growing concern surrounding the reliability of the SLN to accurately predict the disease status of the entire nodal basin. In the head and neck region, the complexity and variability of the interlacing network of cervical lymphatics was highlighted by O'Brien et al who showed a 34% discordance rate between the clinical prediction of lymphatic drainage and lymphoscintigraphy findings in 97 cases of

cutaneous melanoma of the head and neck (O'Brien et al. 1995). A recent study from the John Wayne Cancer Institute reported a 'false negative' rate of 8.9%, identifying increasing tumour thickness, the presence of ulceration and head/neck primary tumours as risk factors for the development of recurrence in the presence of a negative node (Gershenwald et al. 1998). A recent study from the Sydney Melanoma Unit showed that up to 30% of patients with lymph node metastases from neck melanomas bypass the nearest node and involve nodes at more distant sites (Pathak et al. 2001), so called "skip metastases".

Numerous studies have evaluated the survival differences between head and neck melanoma versus those of the trunk and extremities and have found that those with melanoma of the head and neck have relatively poorer outcome (Gillgren et al. 2000; Lachiewicz et al. 2008; Thorn et al. 1989).

Specifically in a study involving 51,704 patients with melanoma, 5- and 10-year Kaplan-Meier survival probabilities for scalp/neck melanoma were 83.1% and 76.2%, respectively, compared with 92.1% and 88.7%, respectively, for melanoma of the other sites, including extremities, trunk, face, and ears. They found that patients with melanoma of the scalp/neck had an 84% greater chance of melanoma-related death compared with those with melanomas of the extremity (Lachiewicz et al. 2008). Within this head and neck group, another large population-based study involving 27,097 patients to evaluate tumour location as a prognostic factor in patients with head and neck melanoma (using the Surveillance, Epidemiology, and End Results (SEER) database), showed a 10-year overall survival rate of 56% and a disease free survival rate of 85%, respectively with those patients diagnosed with scalp/neck melanoma having poorer survival versus those with facial melanoma (Tseng and Martinez).

5. Lack of universal adaptation of sentinel node procedure

There has been some international criticism leveled directly at the manner in which MSLT-1 was carried out and the conclusions derived from it (Thomas 2006, 2009). Stated evidence that, tiny sentinel nodal deposits of melanoma have no prognostic relevance and will not progress or disseminate further as determined by the hosts' immune system, is now accruing. Associating a poorer prognosis to these deposits is called prognostic false positivity; which can lead to patients being incorrectly upstaged, undergoing unnecessary completion lymphadenectomy and possibly unnecessary adjuvant therapy (Thomas 2008). The results of the fourth interim analysis of MSLT-I support the hypothesis that prognostic false-positivity is the explanation for the large survival advantage claimed for patients having early lymphadenectomy versus delayed lymphadenectomy. Further detailed analysis of the results of MSLT-1 suggested that the incidence of prognostic false positivity is about 24% in patients with intermediate thickness melanoma and 34% for all patients (Thomas 2008). Further credence is given to this stance from studies confirming that patients with these tiny deposits of nodal melanoma (ie detected by immunohistochemistry alone or <0.1mm in size), have similar prognosis to those who are sentinel node negative (Spanknebel et al. 2005; van Akkooi et al. 2006).

Some believe that a positive sentinel node is likely to be associated with disseminated melanoma deposits elsewhere because they believe that melanoma tumour biology is not predictable. Are these cells released into the circulation, either lymphatic or blood vascular, disseminating widely and beyond? The timing of their manifestation is that which is unpredictable. These disseminated deposits (nodal, in-transit or distant disease) may

become clinically apparent at some point in time, in spite of a positive sentinel node (and subsequent regional lymphadenectomy). Thus, assessment histologically of the sentinel node and the extent or number of lymph node deposits does not confer any additional benefit to patients, apart from the fact that they have melanoma deposits outside of their primary site and that in time they are at greater risk of further distant disease.

Some experts argue against the routine use of SLNB as they feel that it is associated with an increased risk of in-transit disease, however this has been readily refuted by evidenced based study who showed that there was no significant difference in the rate of in-transit metastasis between patients who had WLE alone (4.9%) and those who had WLE + SLNB (4.5%) (Pawlik et al. 2005).

Clinicians opposed to the use of the sentinel node will argue that in spite of its' prognostic importance, there is still no clear evidence to support a direct survival advantage from the procedure alone and, that no effective adjuvant therapy (including vaccines, combinations of chemotherapeutic agents, immunostimulants, cytokines and growth factors) has been heretofore discovered and subsequently proven to be of clear benefit (unequivocally prolongs overall survival) in clinical trials to date for these patients (Otley and Zitelli 2000; Thomas and Patocskai 2000; Eggermont and Gore 2007; Sabel and Sondak 2002; Spitzer 2002).

Confusion remains whether early completion lymphadenectomy imparts a survival advantage when compared directly to those patients who wait until clinically occult nodal metastatic disease becomes apparent. Some authors site compelling evidence that the assessment of the sentinel node in melanoma is of no benefit whatsoever, does not alter the course of melanoma that has metastasised and should be immediately abandoned from current practice (Medalie and Ackerman 2004). There is growing concern surrounding the reliability of the SLN to accurately predict the disease status of the entire nodal basin. Specific problems have been identified in the head and neck region where the complexity and variability of the interlacing network of cervical lymphatics was highlighted by O'Brien et al who showed a 34% discordance rate between the clinical prediction of lymphatic drainage and lymphoscintigraphy findings in 97 cases of cutaneous melanoma of the head and neck (O'Brien et al. 1995).

Regional nodal failures in melanoma patients following a negative SLNB are not common. Various reasons have been suggested as to why SLNM fails which include (1) when the primary lesion is overlying the draining lymph node basin, (2) learning curve of the performing surgeon / pathologist / nuclear medicine staff, (3) inappropriately high radioactivity level, (4) movement of the dye into the second or non-sentinel lymph node and (5) incorrect injection technique of the dye. It has been suggested that at least a 30 person learning curve be recommended for a surgeon performing these cases (Morton et al. 1999). Patients undergoing SLNM for cutaneous melanoma should be managed via a multi-disciplinary team and the overall success rate of the procedure may be attributable in some part to this.

It is also well recognised that 11-12% (Gadd et al. 1999; Gershenwald et al. 1998) of patients whose sentinel node apparently doesn't harbour cells of melanoma subsequently develop signs of metastatic melanoma disease and conversely patients whose sentinel node does harbour metastatic cells may succumb to their illness in months or they may survive for decades. This high figure does little to add confidence to the therapeutic benefit of sentinel node biopsy.

6. Other emerging strategies

In an ideal oncological world, intra-operative evaluation of the sentinel node for metastatic disease allows a simultaneous completion lymphadenectomy if a positive deposit is immediately identified, preventing the higher costs and additional morbidity associated with a second operation. There is evidence that the sentinel node biopsy procedure is cost-effective compared to wide excision alone (Morton, Howard, and Thompson 2009). Here we discuss some of the emerging strategies addressing this specific issue.

Frozen section analysis of the lymph node was studied in 368 patients with primary cutaneous melanoma ≥ 1 mm or Clarks level IV. 20% (74/368) of sentinel nodes were identified by traditional H&E and immunohistochemical stains, and further frozen sectioning only identified the metastases in 59% (44/74) of these patients (Stojadinovic et al. 2002). The SLN was the only positive lymph node in 86% (64/74) of patients. Additional positive non-SLNs were identified in 10 of 74 patients (14%). This low sensitivity was confirmed in other subsequent studies (Koopal et al. 2000; Tanis et al. 2001). The authors do suggest the potential use of frozen sectioning of a sentinel node found within the parotid gland or neck, thereby reducing the risk of facial and spinal accessory nerve injury during a second operative procedure. Frozen sectioning is a technically difficult procedure to perform and results in freezing artefact of the lymph node, rendering it unsuitable for further analysis.

Another recent development concerning the sentinel node is imprint cytology, which involves bisecting the node, imprinting it and subsequently staining the relevant sections. One study involving 93 patients showed that imprint cytology had a sensitivity and specificity of 38% and 100% respectively (similar figure to that of frozen section) (Creager et al. 2002). Even though it is easier to perform this procedure and without subsequent damage to the lymph node, the low sensitivity severely limits its use in the routine evaluation of the sentinel node. Other algorithms of histological factors concerning the primary tumour have also been developed (vertical growth phase, tumour infiltrating lymphocytes, mitotic rate and thickness) (Kruper et al. 2006). These are able to stratify patients into high and minimal risk for sentinel node disease based on the analysis of their primary lesion, and upon validation, these models could possibly provide a clinically useful tool for making treatment decisions (remove the use of the sentinel node procedure in those with minimal risk), aid in assessing patient risk, and for planning and analyzing clinical trials (Soong et al.).

Some molecular biology techniques for the staging of regional lymph nodes have yielded promising results (Wang et al. 1994). Wang et al, using real-time PCR to identify tyrosine messenger RNA for the detection of micrometastatic lymph node disease, showed that this was a highly sensitive and clinically applicable method to detect micrometastases (tyrosine messenger RNA is found almost exclusively in melanocytes). Furthermore, studies using reverse transcription and polymerase chain reaction to determine tyrosinase mRNA in peripheral blood, (which indicates the presence of circulating melanoma cells), showed that this may be a promising serum marker for melanoma staging and for predicting recurrence, prognosis and response to immune therapy (Brossart et al. 1993). The same group found that the amount of circulating tumour cells correlates with the tumour burden and that in patients with regression of melanoma metastases after immunotherapy, a decrease of the amount of tumour cells in the peripheral blood was observed (Brossart et al. 1995), associating the rate of positivity with stage of the disease.

There is accumulating evidence to support the use of targeted ultrasound assessment of the regional nodal basin at the time of diagnosis of the primary tumour, thus enhancing routine

clinical palpation. It is possible to identify deposits as small as 3-4mm (Bafounta et al. 2004). This does not take into account those nodal deposits below that diameter ie <3mm. Voit et al showed that specific features on preoperative ultrasound (peripheral perfusion, balloon shape and loss of central echoes) and fine needle aspiration cytology can identify 65% of sentinel node metastases and thus reduce the need for surgical procedures on the sentinel node, allowing those patients identified to proceed directly to completion lymphadenectomy (Voit et al.). However these results must be considered with caution. Ultrasound is heavily user-dependent. Given the fact that some patients will present with micrometastatic deposits in their regional nodes that will not be identifiable on ultrasound lowers its' sensitivity. Therefore, a negative ultrasound is not a reliable substitution for biopsy and subsequent histopathological examination of the sentinel node.

MicroRNAs (miRNAs) are non-coding short ribonucleic acid molecules that are post-transcriptional regulators that bind to complementary sequences in the three prime untranslated regions of mRNAs, leading to gene silencing. MiRNAs are present in human plasma in a very stable form that is protected from endogenous RNase activity (Mitchell et al. 2008). There has been a recent surge in reports demonstrating the use of miRNAs as modulators of gene expression and their potential role as both diagnostic and prognostic markers in many malignancies (Iorio and Croce 2009; Osaki, Takeshita, and Ochiya 2008). This is also true for malignant melanoma, where it has been shown that serum levels of miR-221 were significantly increased in melanoma patients, differentiated between in situ and invasive disease, were useful in evaluating tumour progression and monitoring patients during follow-up and that levels of miR-221 correlated with tumour thickness (Kanemaru et al.).

7. Conclusion

In spite of a lack of definitive evidence associating a positive SLNB result and increased survival rate, the routine use of this novel approach is justified in patients presenting with primary cutaneous malignant melanoma because it provides valid and reliable staging information about the regional nodes using a minimally invasive technique, allows for regional disease control in the presence of a positive sentinel node and increases the potential for cure in patients with at least intermediate thickness disease. Cumulative evidence from MSLT-1, ELND trials and large retrospective trials mentioned above support the view that survival is better for patients with clinically occult sentinel node deposits.

8. References

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Management of Head and Neck Melanoma

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1. Introduction

The incidence of melanoma in New Zealand (NZ) is one of the highest in the world: The rate in Caucasians is approximately 50 per 100,000, while the incidence in the NZ Maori population and the NZ Polynesian population is much lower contributing 15% and 7% of the total number of melanoma reported annually (Shaw, 2008). The primary author has twenty-six years' Consultant Surgical experience with melanoma surgery. Our database goes back to 1984 and includes 3,500 patients, of which 550 patients have been managed for head and neck melanoma. We have included all patients with either head and neck primary melanoma or regional melanoma involving cervical regional nodes or parotid nodes managed in the Auckland area 1984-2005. No patients have been excluded. These Head & Neck melanoma (H&NMM) data have never been published in the world literature. We reviewed our head and neck melanoma data base and this constitutes the basis of this study.

2. Background & methods

The head and neck area is a preferred site for melanoma; 9% of the body's surface area but 15% of the total melanoma distribution (Douglas & Shaw, 1987)

The outcome is probably less favourable in the head and neck area than in limbs or trunk (Douglas & Shaw, 1987) (Fisher & O'Brien, 2002). In addition, some head and neck sites are less favourable than others for example scalp, mucosal lesions tend to be less favourable than facial lesions (Douglas & Shaw, 1987; Fisher & O'Brien, 2002), further it is unclear whether melanomas from the trunk metastasising to cervical lymph nodes are more or less favourable than melanomas spreading to regional nodes with a head and neck primary.

Over the past 20 plus years for lesions < 1 mm in thickness we have used a 1 cm margin for lesions, > 2 mm thickness a 2 cm margin and for lesions between 1 and 2 mm thickness a margin of between 1 and 2 cm. The approach for thin lesions has been based on the work of Umberto Veronesi and colleagues (Veronesi et al, 1998), while our approach for thicker lesions has been validated in a prospective study by Merion Thomas and colleagues (Thomas et al, 2004).

Prior to the advent of sentinel node biopsy regional disease was treated with a therapeutic node dissection after positive fine needle aspirate cytology (FNA). Most commonly this was a selective neck dissection. Since the advent of sentinel node biopsy patients with a positive sentinel node have all been treated with either a selective or modified neck dissection depending upon the site of the primary lesion. After neck dissection virtually all patients

were offered radiotherapy and this was accepted by around half the patients. Nodal disease was classified N0 if no nodes were involved, N1 if one node was positive, N2 if 2-3 nodes were involved, and N3 if 4 or more nodes involved.

Survival was calculated using Kaplan Myer analysis while comparison between patient groups was performed using Chi squared analysis.

Questions asked of our data-base included assessment of :

- a. Anatomical-site, sex, and primary thickness on outcome
- b. Significance of local and regional recurrence on outcome
- c. Determine extent of neck dissection required as a function of primary anatomical site
- d. Determine effect of adjuvant radiation following neck-dissection on regional re-recurrence and survival
- e. Assess the outcome for patients with primary site that is:
 - i. outside head and neck area
 - ii. desmoplastic subtype and
 - iii. mucosal subtype

3. Results

3.1 Anatomical distribution and thickness of primary lesions

The commonest H&N primary anatomical site was face (52%), followed by scalp (19%), neck (17%), ear (9%), and mucosal lesions (3 %).

37% of primary lesions were < 0.75 mm thick, 14% were 0.76-1.5 mm thick, 27% were 1.5 - 4.0 mm thick, and 23% were > 4.0 mm thick.

Predictably, as in other parts of the body, the thickness of the primary lesion governed outcome see Figure 1.

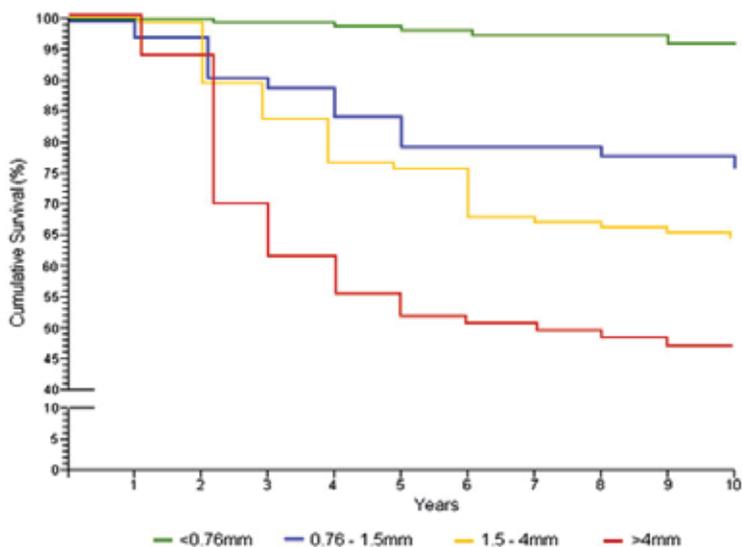


Fig. 1. Effect of Primary Thicknesses on Survival – Auckland

The sex incidence was higher in males than females and is summarised in Table 1:

Trunk primary lesions metastasising to cervical nodes, and scalp lesions, were significantly more common in males ($p < 0.05$, and $p < 0.03$ respectively). Ear lesions were almost twice as common in males as in females.

	Male	Female	Ratio
Face	140	126	1.1
Neck	49	40	1.2
Ear	28	15	1.9
Trunk	15	4	3.8*
Scalp	75	10	7.5 **
Total	307	195	1.6

Table 1. Sex Incidence Head & Neck Melanoma - Auckland

The effect of anatomical site on survival is shown in fig 2. Survival for facial lesions is significantly better than for neck lesions ($p < 0.05$) and for Scalp & ear lesions ($p < 0.01$).

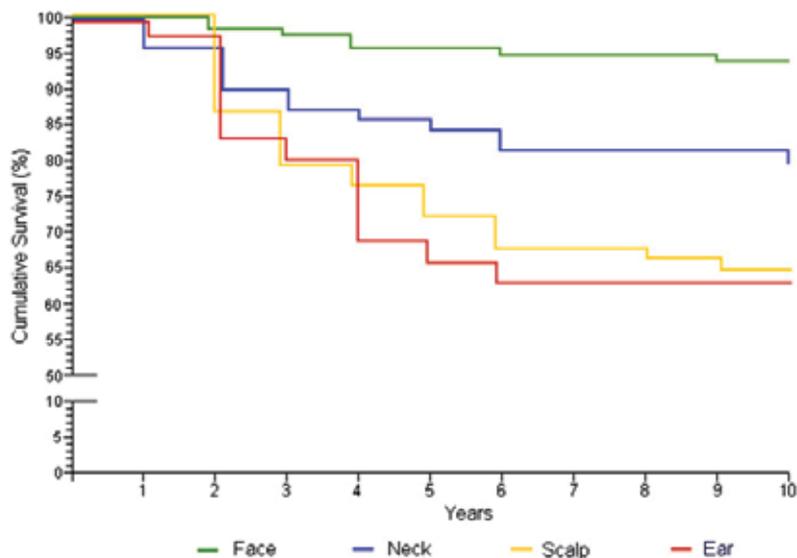


Fig. 2. Effect of Anatomical Site on Survival - Auckland

Local recurrence (LR) occurred in 5% for patients with facial, neck and ear lesions versus 13% for scalp lesions ($p < 0.05$) and 50% for mucosal lesions ($p < 0.01$). Local recurrence became progressively more common with increasing thickness of the primary lesion: see Table 2.

Thickness mm	% Local Recurrence	Fraction
< 0.76	5%	8/168
0.76 - 1.5	11%	7/62
1.5 - 4.0	10%	11/112
> 4	20%	20/99
Overall	10%	46/441

Table 2. Effect of Thickness on Local Recurrence - Auckland

The pie graph (Fig 3) outlines the most likely reasons for local recurrence and their relative frequencies.

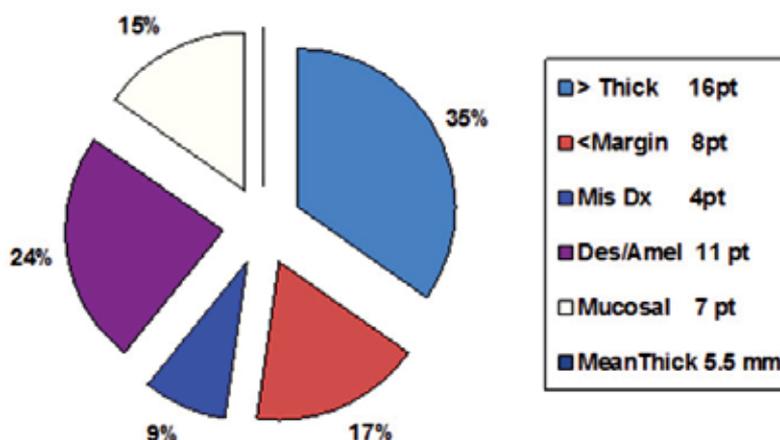


Fig. 3. Factors Associated with 46 Local Recurrences - Auckland

Local recurrence had a major impact on outcome in particular there was a significant increase in both the development of regional disease and also death from disease in those patients who developed LR compared with those who did not develop LR. see Table 3

No Local Recurrence		Local Recurrence	
Regional Nodes	26% (97/425)	Regional Nodes	46%*21/46***
Dead of Disease	25% (99/425)	Dead of Disease	72%*** (33/46)****

Table 3. Local Recurrent & Outcome - Auckland

The impact on survival of either local recurrence or regional recurrence was almost identical: see Figure 3.

Regional disease was a function of thickness of the primary with 5% of patients developing regional disease with primary lesions < 0.75 mm. 25% of patients with primary lesions 0.76 – 1.5 mm thickness developed regional disease and 35% of patients with melanomas over 1.5 mm thickness developed regional disease.

The timing of regional disease was a function of the thickness of the primary melanoma. Patients who had regional disease at initial presentation had a mean primary thickness of over 6 mm, while patients who presented with progressively thinner primary lesions developed regional disease progressively later. see Table 4.

Timing of Nodes	10 Thickness	No of Nodes
@ Diagnosis	6.2 +/- 1.2 mm	2.1
@ 1-12 mths	2.8 +/- 0.4 mm	3.1
@ 12-48 mths	1.9 +/- 0.3 mm	2.8
@ 48 mths +	1.3 +/- 0.5 mm	2.2

Table 4. Timing of Nodal Disease v 1⁰ Thickness - Auckland

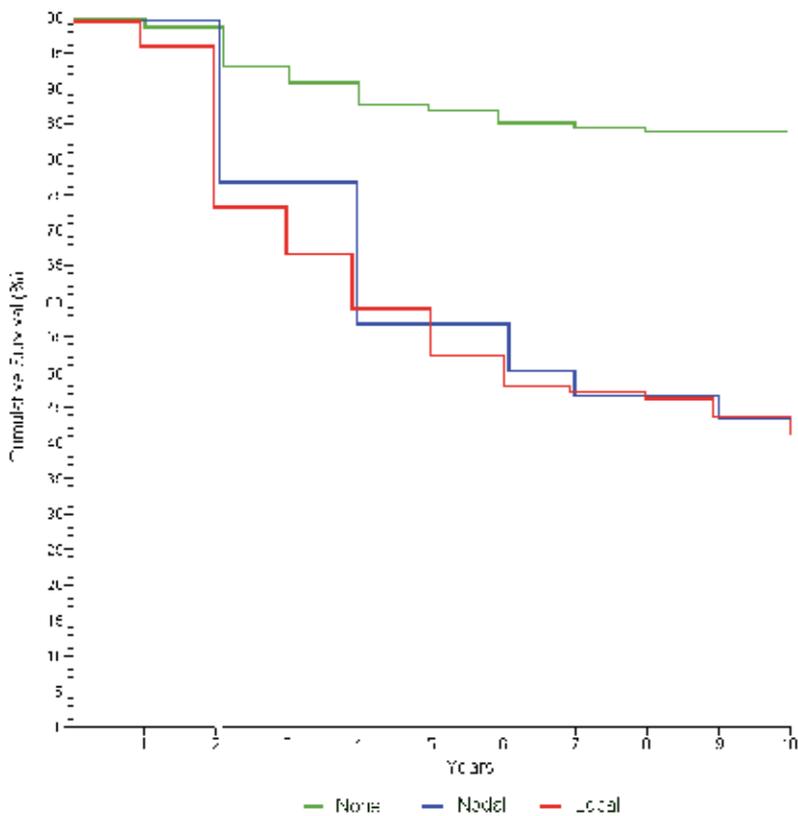


Fig. 3. Influence Local or Regional Recurrence on Survival - Auckland

Survival following the diagnosis of regional disease was not influenced by disease free interval. Although patients who had longer disease free intervals between initial diagnosis and the development of regional disease lived longer overall, once regional disease occurred the survival curves were not significantly different. See fig 4.

The sites of Regional Disease as a Function of anatomical site of the primary lesion are summarised in Table 5. The commonest sites of regional disease were level 2 nodes and parotid nodes.

	Ear n=14	Face n=41	Scalp n=39	Neck n=29	Total n=123
Parotid	64%	46%	31%	10%	38%
Level 1	-	11%	7%	21%	9%
Level 2	50%	29%	30%	90%	50%
Level 3/4	7%	5%	20%	67%	25%
Level 5	-	5%	28%	67%	25%
Sub-occipital	-	-	8%	7%	4%
Post-auricular	-	-	18%	7%	6%

Table 5. Regional Disease v Anatomical Site of Melanoma - Auckland

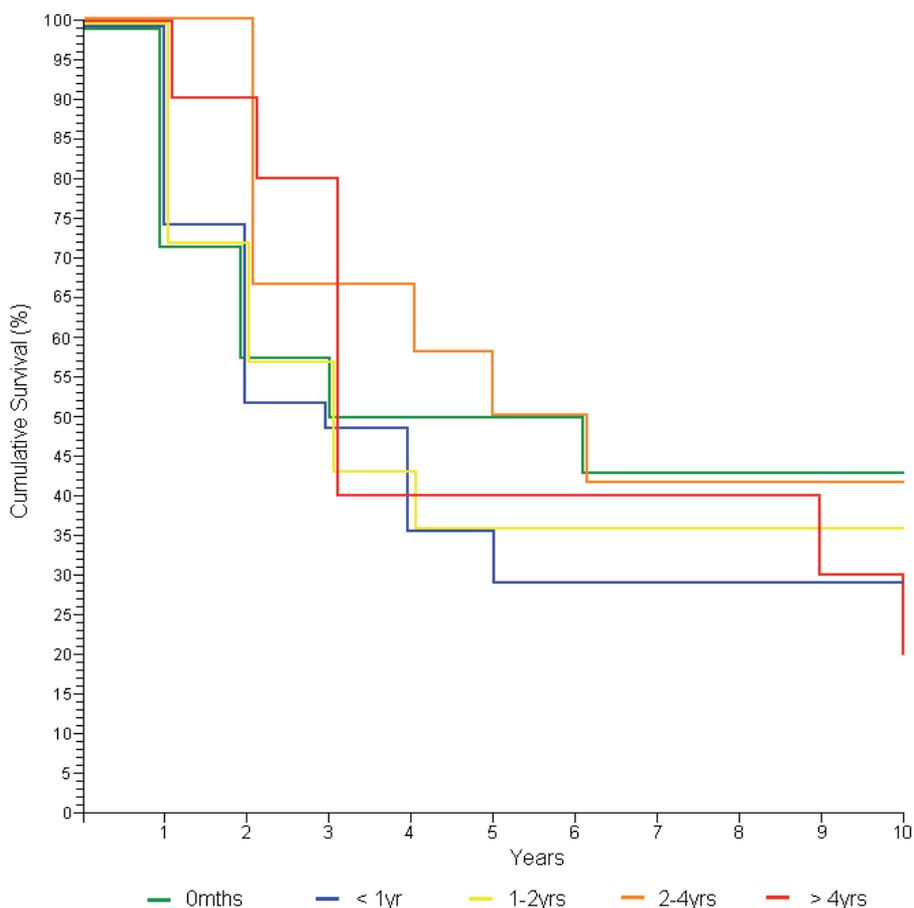


Fig. 4. Survival Node +ve Patients from Onset of Regional Disease - Auckland

3.2 Sentinel node studies

Our Sentinel node data were in general accord with the above data regarding site of regional disease as a function of anatomical site of the primary melanoma with Level 2 and Parotid being the commonest sites of involvement. The unusual findings from these studies were:

1. Bilateral or contralateral sentinel nodes in patients with lesion near the midline.
2. The frequent incidence of multiple sentinel nodes (up to 5)
3. The unpredictability of upper trunk lesions, for example spreading to contralateral Level 1.

See Discussion for detailed analysis of sentinel data with world literature.

3.3 Survival as a function of regional disease

Patients without regional disease (stage No) had 90% 5 year survival. Patients with only one node involved (stage N1), had 70% 5 yr survival, patients with stage N2 disease (2-3 nodes involved) had 57% 5 yr survival, while N3 patients with 4 or more nodes involved had 37% 5 yr survival. See Fig 5

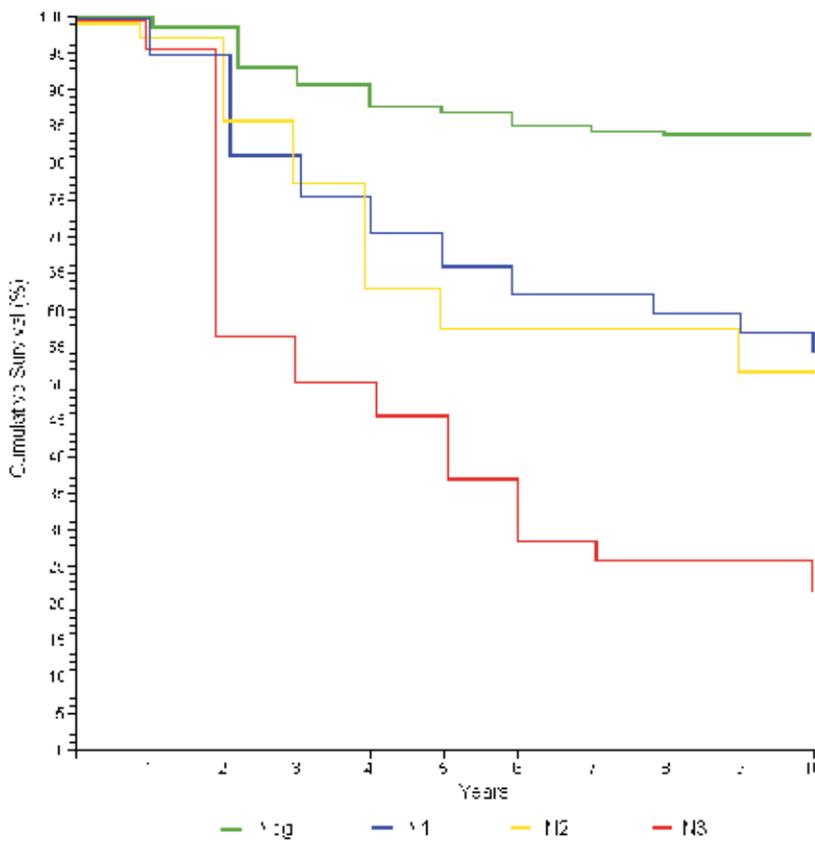


Fig. 5. Stage of Regional Disease & Survival – Auckland

3.4 Regional failure after neck dissection and the impact of radiation

Regional failure was dependent on the N status of the neck. The rate of failure in patients with N1 disease was 5%, N2 disease 17% and N3 disease 22%. These rates of regional failure were all decreased with radiation and these reached statistical significance for patients with N3 disease and for regional disease overall. See Table 6.

	% Failure no RT		% Failure with RT	
N1	11%	1/19	3%	1/31
N2	25%	4/16	9%	2/22
N3	38%	8/21	10%*	2/20 p < .04
Overall	28%	13/46	7%***	5/71 p < .002

Table 6. Influence of RT on Recurrence after Neck Dissection

Although adjuvant radiation significantly decreased regional recurrence after neck dissection adjuvant radiation had no impact on survival: The Kaplan Meyer curves for survival after neck dissection alone versus neck dissection with radiation the curves were virtually super-imposed. See Fig 6

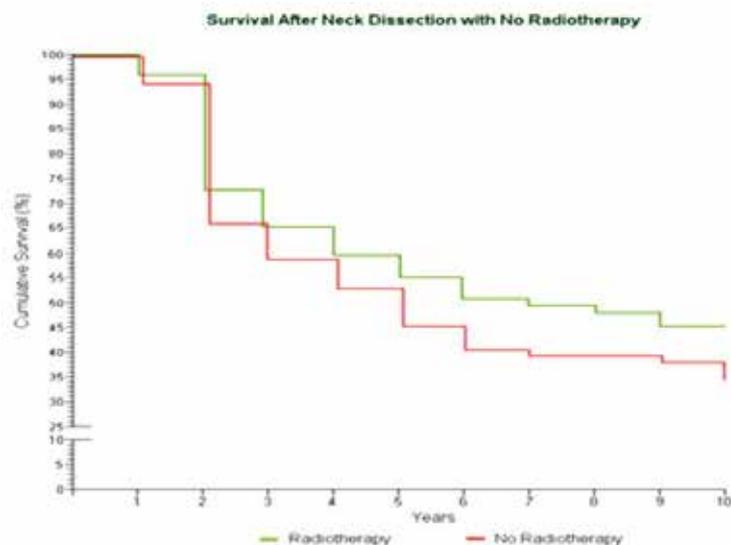


Fig. 6. Survival after neck dissection with and without Adjuvant radiation

3.5 Less common situations

3.5.1 Primary site unknown

There were nine patients in this group all with stage N2 or N3 disease with a 35% 5 year survival which was virtually identical when compared with the 5 year survival for the group of patients with N2 and N3 disease who had a known primary.

3.5.2 Cervical regional disease from primary site on trunk

There were 19 patients in this group, 14 had axillary and neck disease, 5 had neck disease alone. The mean number of nodes involved was was 11 (range 3-48). Neck Levels most commonly involved were:

Level 5: 14 patients

Level 4: 6 patients

Level 3: 4 patients

Level 1 or 2: 4 patients (including 1 patient with contralateral Level 1 disease)

The outcome was very poor with 15/19 patients dead of disease at five years. See Fig 7

3.5.3 Desmoplastic neurotropic melanoma

There were 19 patients in this group. The lesions were thick with a mean thickness of 3.5 mm. When the desmoplastic patient group were compared with patients with equivalent thickness lesions that were not desmoplastic: the local recurrence rate was higher in the desmoplastic group at 26% versus 7% ($p < 0.04$), while regional recurrence was lower in the desmoplastic group at 22% versus 47%. Death from disease was virtually identical in the two groups See Table 7.

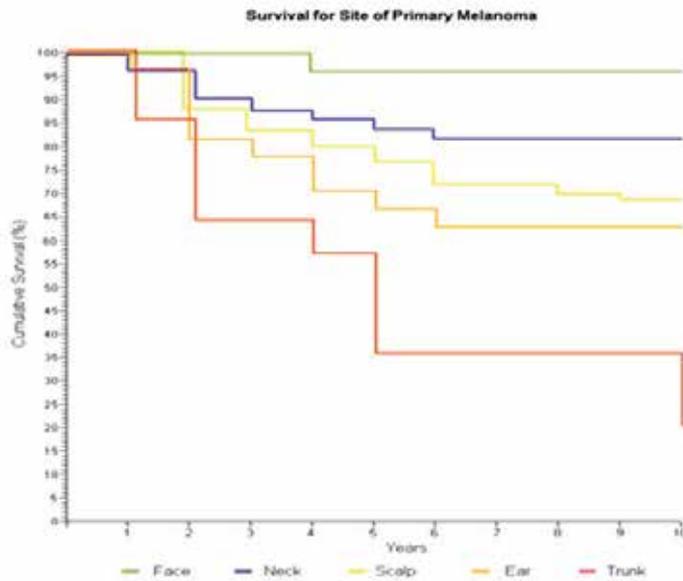


Fig. 7. Survival of Patients with Trunk Primary versus primary lesion in the Head and Neck

	Non Desmoplastic	Desmoplastic
Median Thickness	3.5 mm	4.1 mm
Local Recurrence	7% (20/261)	26%*** (9/19)
Regional Nodes	47%* 107/261)	22%* p.06 (4/19)
Dead of Disease	41% (8/19)	42% (95/261)

Table 7. Desmoplastic v Non-Desmoplastic Melanoma – Auckland - Stratified for Equivalent Thickness

3.5.4 Mucosal melanoma

There were 14 patients in this group. The commonest sites were conjunctival, oral and nasal. There were poor outcomes here even for very thin lesions. Seven of the 14 patients developed local recurrence and 5/14 were dead of disease at 5 years despite a mean thickness of only .3 mm.

4. Discussion

Our study is one of the largest studies of Head & Neck melanoma reported from a single centre. The single greatest experience is however from the Sydney Melanoma Unit (De Wilt et al, 2004; Fisher & O'Brien, 2002; Thompson et al, 2005), and we have compared and contrasted our experience with theirs along with various centres worldwide.

The data from Fisher S.R & O'Brien, C.J., 2002 are similar to ours with respect to anatomical site, sex-incidence. In addition outcome versus anatomical site were similar to reports of others with face and neck lesions having a more favourable outcome than scalp and ear. In addition the reason for these differences is largely explained on the basis of the face & neck lesions being thinner and therefore having a better outcome. Further, although H&NMM

was more common in males the two sites where the incidence was almost the same for males and females (Face and Neck) the outcome was better. We have previously shown that for melanoma overall when all sites are pooled the incidence in males and females is similar (Jones et al, 1999) and that overall males tend to have poorer outcomes than females especially older males (Jones et al, 1999; Shaw, 2008). Trunk and scalp lesions were significantly more common in males in our study: we have previously shown the highest incidence of melanoma in males is on the back (Jones et al, 1999), Scalp melanoma is most common in bald patients and there are more bald men than women (Douglas & Shaw, 1987; Jones et al, 1999).

The outcome for patients with desmoplastic melanoma (DM) is similar to that reported by Quinn and colleagues from the Sydney Melanoma Unit (Quinn et al, 1998). In particular DM is associated with a relatively high incidence of LR coupled with a relatively low incidence of regional disease and when compared to non DM melanoma patients a similar risk of death. The relatively low rate of regional disease has some implications when considering the performance of sentinel node studies in patients with DM. Our data indicating that the outcome of patients with regional disease and primary site unknown is similar to patients with regional disease and primary site known when stratified for similar stage of regional disease are in general agreement with the literature (Santini et al, 1985).

Local recurrence (LR) in H&NMM has been reported to be more common than for limb melanomas (Douglas & Shaw, 1987; Jones et al, 1999; Ng et al, 2001). Our current findings are in agreement with this in a general sense, with the pie chart summarising the individual main causative factors, namely thickness of the primary, inadequate margin, unfavourable histological sub-type. We have previously documented the effect of tumour thickness and inadequate margin on local recurrence (Ng et al, 2001). In addition the studies by Balch and colleagues (Balch et al, 2001) have highlighted that tumour thickness is very reliable and an independent predictor of both recurrence and survival.

One sub-group where this rule of thickness being a reliable predictor of outcome is not useful is in patients with mucosal melanoma; the highest incidence of which is in the head and neck region (Tomicic & Wanebo, 2003). In our patients despite the medial thickness being only 1.3 mm the rates of recurrence and death from disease were markedly higher than for non- mucosal lesions of similar thickness. A variety of authors have underlined the poor prognosis associated with mucosal melanoma irrespective of either conservative or radical treatment involving surgery alone or combined with radiation (Tomicic & Wanebo, 2003), but the fact that even very thin lesions tend to recur appears to be relatively under-reported.

The implications of LR are substantial with approximately double the incidence of regional disease and approximately a three- fold risk of dying of melanoma. Further, the impact of LR and regional recurrence on survival were virtually identical. These findings are similar to those reported by O'Brien and colleagues (Fisher & O'Brien, 2002)

The regional nodes of significance when managing H&NMM are outlined in Fig 8. See below: In particular the nodes as classified by Memorial Sloan Kettering Cancer Centre (Shah et al., 1991) involving levels 1-5, along with intra-parotid nodes coupled with the post-auricular nodes and the Sub-occipital nodes highlighted by the SMU (De Wilt et al, 2004). In addition the axillary nodes are important especially for upper trunk lesions and lower neck lesions. The importance of primary lesions from trunk regional to the neck, and neck lesions regional to the axilla(e) has been relatively understudied but following the widespread use of sentinel node studies a far better understanding of H&N regional

lymphatics has been developed, especially by Morton and colleagues at the John Wayne Cancer Centre (Morton et al, 2006) and Thomson and colleagues in Sydney Australia (De Wilt et al., 2004; Thompson et al., 2005).

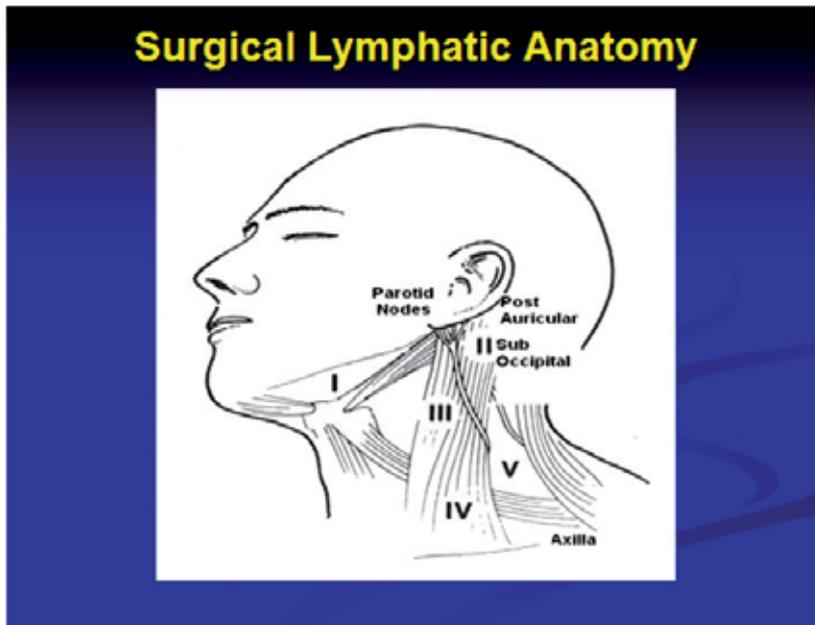


Fig. 8. Surgical Lymphatic Anatomy

Timing of regional failure in our study was a function of primary tumour thickness with patients who had regional disease at initial presentation had a mean primary thickness of over 6 mm, while progressively thinner primary lesions went on to be associated with region disease of several years: 3 mm lesions tending to recur regionally inside 1 yr, 2mm lesions recurring regionally most commonly in 1-4 yrs, and lesions 1-2 mm thick recurring most commonly later than 4 yrs. These findings are similar to those reported by O'Brien and Fischer (Fisher & O'Brien, 2002) and underline the capacity of even relatively thin melanomas to recur many years after initial diagnosis (Thompson et al., 2005). These finding also fit well with the findings of Balch and colleagues in 2001 (Balch et al., 1985). After analysing 10 yr survival data for over 15,000 melanoma patients with lesions in all anatomical sites, the 10 yr mortality increased steadily as a smooth curve as a function of primary thickness from approximately 20% for 1 mm lesions through to 70% mortality for lesions 10 mm thick. See Fig 9 below

For some malignancies disease free interval is an important prognostic factor (Fisher Fisher & O'Brien, 1998), however for melanoma the situation is less clear (Thompson et al., 2005). In our study although patients who developed regional progressively further along the course of their disease had a relative long absolute survival, once regional disease occurred the survival data curves from that time on were not significantly different between the various groups.

One very important but contentious issue in the surgical management of H&NMM patients is the extent of neck dissection which should be performed for regional disease as a function

of the anatomical site of the primary melanoma. Shah and colleagues in 1991 (Shah et al., 1991) concluded that virtually all neck nodal levels along with the parotid nodes should be dissected in virtually all patients with H&NMM. Their data were based on therapeutic neck dissections for FNA diagnosed palpable regional disease. Their data are presented in modified format in Table 8

Site of Nodal Involvement v Anatomical Site						
Shah et al., Amer J Surg, 1991						
	Face	Ant Scalp	Ant Neck	Post Neck	Post Scalp	Ear
Parotid	44%	100%	33%	-	29%	83%
L1	48%	57%	13%	-	47%	27%
L2	48%	65%	50%	50%	60%	73%
L3,4	12%	50%	28%	25%	27%	41%
L5	20%	57%	-	30%	20%	27%

Table 8. Regional H& N Melanoma v Anatomical Site, Shah et al., Amer J Surg 1991

From the Shah data (Shah et al., 1991) one would conclude that virtually all patients with regional H&NMM require a Level 1-5 neck dissection and resection of parotid nodes. The exceptions being that posterior neck does not require parotidectomy (P) or dissection of Level 1, while anterior neck primary while requiring parotidectomy does not require dissection of Level 5 nodes, and only occasionally will level 1 nodes be involved.

Another major study addressing sites of regional disease as a function of anatomical primary melanoma site is that from the Sydney Melanoma Unit (SMU) (De Wilt et al., 2004). Their approach was a different one whereby 918 sentinel nodes were mapped in 362 patients with H&NMM. A modified assessment of their data is presented in Table 9 below.

The conclusions from the Sydney data are in general agreement with those of Shah and colleagues (Shah et al., 1991) whereby:

1. Ear primary requires P, and Levels 2-5 neck dissection, but in contrast to Shah (Shah et al., 1991) level 1 would not require dissection
2. Face primary requires P and Levels 1-4, but in contrast to Shah (Shah et al., 1991) who found 20% of facial lesions involved L5
3. Anterior scalp requires P and Levels 1-4 and possibly L5 also as 10% chance of involvement at SMU versus 57% involvement at MSKCC.
4. Anterior neck requires Levels 1-5 but not P while Shah (Shah et al., 1991) found 33% had parotid involvement but no L5 disease

918 Sentinel nodes in 362 Melanoma Patients								
SMU Ann Surg 2004								
	Parotid	L1	L2	L3 / 4	L5	SO	PA	Axilla
Ear	36%	-	90%	12%	27%	-	21%	-
Face	57%	48%	70%	20%	-	-	-	-
P Scalp	14%	-	47%	18%	59%	39%	32%	-
C Scalp	50%	-	70%	10%	30%	20%	50%	-
C Neck	17%	20%	80%	24%	40%	-	-	-
A Scalp	80%	-	90%	-	10%	-	35%	-
A low Neck	-	-	28%	21%	77%	-	-	43%
A up Neck	-	13%	63%	38%	25%	-	-	-
P low Neck	-	-	14%	28%	100%	18%	-	23%
P up Neck	11%	-	72%	15%	56%	16%	-	-

Table 9. 918 Sentinel Nodes in 362 Melanoma Patients

5. Posterior neck requires Levels 2-5 neck dissection with P only rarely required, concurring with while Shah (Shah et al., 1991)
6. Posterior scalp requires levels 2-5 neck dissection with only 14% chance of P being required, while Shah (Shah et al., 1991) found level 1 involved in 47% & and Parotid in 29% of patients.

The SMU data (14) also highlight the importance of the post-auricular and the Sub-occipital nodes especially for patients with Ear, Scalp, and posterior neck lesions. These were not assessed in the Shah data (Shah et al., 1991).

Our own data agree with Shah (Shah et al., 1991) that: L1 does not usually require dissection for ear primary

Our data are in agreement with SMU 14(15) :

1. Face does not usually require dissection of L5
2. L1 only rarely involved aside from face and neck primary lesions

Our data are in agreement with both MSKCC and SMU that neck lesions require a L2-5 dissection and occasional inclusion for dissection of L1 and P depending on the site of the neck primary. Our data also highlight the importance of upper trunk lesions spreading to cervical nodes, and the fact that these lesions have an especially bad prognosis as most patients have N2 or N3 disease often with involvement of both cervical nodes and one or more axilla. The importance of trunk lesions involving neck nodes, and the associated bad prognosis has been relatively understudied in the literature. The principal levels involved by trunk primary melanoma are levels 5 followed by levels 4, but occasionally levels 3,2,and 1 are involved. In addition for mid line lesions especially contralateral disease in unexpected sites may occur. In our study the outcome for trunk lesions that metastasised to cervical nodes either alone or in combination with axillary disease is markedly worse than for acral lentiginous melanoma, a known poor prognosis subtype which we have reported on previously (Koea & Shaw, 1988)

In order to provide a guideline for management of the neck in H&NMM we have modified the figure from O'Brien and colleagues (O'Brien, 1999) to provide some general rules regarding extent of neck dissection as a function of primary anatomical site. See Fig 9

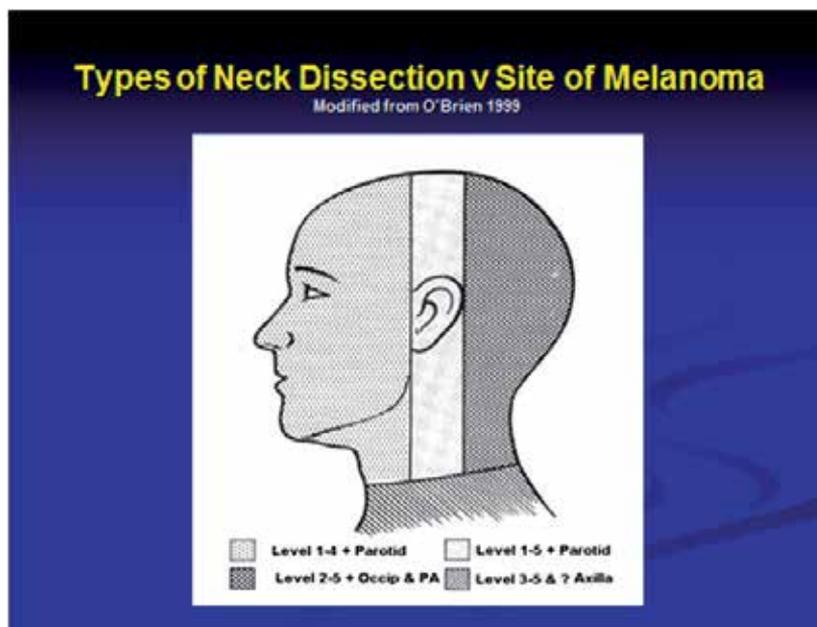


Fig. 9. Types of Neck Dissection v Site of Melanoma

There are a number of provisos to be incorporated with this figure based on our findings and those from SMU (15) and MSKCC (14). These include:

1. The risk of contralateral and bilateral regional disease with primary melanomas close to midline
2. Level 1 is not often involved but occasionally may be involved from as far away as posterior trunk and rarely bilateral
3. Trunk lesions may require dissection of more than levels 4 and 5, and involvement of one or both axillae must be excluded either by sentinel node study or imaging

Results predicting regional disease levels as a function of anatomical site of primary may be different when data from sentinel node studies are compared with studies from therapeutic neck dissection data. Also the extent of the neck dissection performed is also important for example; in our study selective dissection was frequently used in our patients whereas in the Shah (Shah et al., 1991) data many patients underwent either conservative or radical neck dissection. In addition spread of melanoma cells along involved lymphatic channels may be different compared to the distribution of tracer as part of a sentinel node study in a clinically uninvolved neck.

Survival as a function of extent of regional disease in our study is similar to what has been reported by others (Balch et al., 2001; Fisher & O'Brien, 2002; O'Brien, 1999). One common finding across centres is the dramatic drop-off in survival between N1 -N2 disease and N3 disease. The reason for this being that the maximum number of nodes involved in N2 patients is 3 while some patients with N3 disease may have 20 or more nodes involved. See Table 10

	NZ	UAB	SMU	Duke	MDA	Overall
N1	64%	53%	40%	-	39%	50%
N2	57%	48%	-	-	39%	49%
N3	36%	27%	12%		23%	25%
Overall	48%	43%	38%	45%	35%	42%

Table 10. Regional Disease 5y Survival

NZ: New Zealand

UAB: University of Alabama

Duke: Duke University

MDA: MD Anderson Cancer Clinic, Texas

In more detail the results of O'Brien and others (20) are summarised in Table 11 below:

<ul style="list-style-type: none"> ■ 152 Therapeutic neck dissections ■ 28% Re - recurrence in neck ■ 34% 10 yr Survival ■ 67% if node -ve ■ Postoperative RT: multiple nodes or ECS

Table 11. Neck Dissection for Melanoma - (O'Brien et al., 1992)

The role of adjuvant radiation to minimise recurrence after neck disease is debated in the literature. We offered the majority of patients adjuvant radiation while many clinicians offer radiation only for patients with multiple nodes or if extra-nodal spread is present (eg 20).

While the role of adjuvant radiation will only be totally resolved by a prospective randomised study some important findings emerge from our data. Firstly we were able to show a significant reduction in recurrence in patients who received adjuvant radiation, especially those with N3 disease. O'Brien (O'Brien et al., 1997) was also able to demonstrate a reduction in recurrence but this fell short of statistical significance (just). Our overall recurrence rate of 28% in patients who did not receive adjuvant radiation is similar to the figure reported by O'Brien et al (O'Brien et al., 1997) and lower than many reports (O'Brien et al., 1997). The dramatic decrease in recurrence in our patient both with N3 disease and regional disease overall is in agreement with the findings of Ang and colleagues (Ang et al., 1994) from the MD Anderson unit in Texas. Ang and colleagues (Ang et al., 1994) were able to demonstrate a dramatic reduction in recurrence when neck dissection was followed with adjuvant radiation compared with historic control data where surgery alone was used. Incidentally they were also able to demonstrate that adjuvant radiation also reduction in recurrence following excision of prognostically unfavourable primary melanomas. For many years melanoma was considered to not be a radio-responsive tumour but this is now largely refuted (Mendenhall et al., 2008). The fact that adjuvant radiation decreased regional recurrence in our study while not influencing survival makes good sense from the surgical oncology standpoint. Adjuvant radiation is well known to minimise recurrence following neck dissection for squamous cell carcinoma (Fisher & O'Brien, 1998), and adjuvant radiation has been shown to minimise recurrence following anterior resection for rectal cancer (Fisher & O'Brien, 1998), and also minimise recurrence in some patients following surgery for Breast cancer, but in most of these circumstance this decrease in recurrence does not translate into improved survival.

We have not presented data addressing complications in this present study. We have previously presented a comparison between our complications for node dissection in the neck, the axilla, and groin (Shaw & Rumball, 1990). In that study the complication rate following neck dissection and axillary dissection was similar and in both sites was markedly lower than following neck dissection. In addition the complications associated with the addition of adjuvant radiation was lower in the neck than in the two other sites.

5. Conclusions and recommendations

1. The head and neck area is a preferred site for melanoma and has an unfavourable outcome in comparison to the rest of the body with respect to local recurrence and death from disease.
2. There is a complex lymphatic system and both the performance of sentinel node studies in the head and neck area and their analysis is more complex than in the trunk or limbs. Unpredicted sites of sentinel nodes are more common in the head & neck region than in the rest of the body.
3. The timing of regional disease in H&NMM is important. Patients who had palpable regional disease at the time of initial presentation had an average primary thickness over 6 mm. Patients with progressively thinner primary lesions developed regional disease over subsequent years with the general rule being the thicker the primary the more quickly regional disease developed. Many of these patients were assessed prior to the use of sentinel node mapping and this will probably detect patients likely to recur especially in the first year after initial diagnosis of melanoma. Prolonged disease-free interval does not appear to be a favourable prognostic factor once regional disease has occurred.

4. Management of regional disease can often be accomplished using a selective neck dissection, the regional beds requiring resection can be assessed as a function of the primary site of the melanoma. In particular:
 - a. For anterior scalp and face parotidectomy plus levels I-IV neck dissection.
 - b. For coronal lesions including scalp and ear parotidectomy and levels I-V neck dissection.
 - c. For posterior scalp and posterior neck level II-V neck dissection.
 - d. For trunk lesions with cervical regional disease Level 4 & 5 neck dissection is appropriate, often in association with one or more axillary dissections.
 - e. Recommendations for sites of regional disease that should be resected for various primary melanoma sites are summarised in Fig 7. It is important to note that this is just a guideline as sites of regional disease are unpredictable. In particular upper back lesions involving cervical nodes can occasionally involve as far away as level 1, while primary lesions close to the midline (either head and neck or upper trunk primary) have a tendency to involve either contralateral nodes or bilateral nodes.
 - d. The post -auricular nodes and sub- occipital nodes have to be considered in for patients with posterior scalp, coronal scalp and ear lesions and to a lesser extent with patients with posterior neck lesions.
5. Desmoplastic melanoma, mucosal melanoma, and melanoma from trunk metastasising to neck have a relatively poor prognosis. Desmoplastic lesions tend to have relatively low rates of regional disease and relatively high rates of local recurrence resulting in survival similar to non-desmoplastic melanomas. Trunk melanomas metastasising to the neck usually represent N3 disease (occasionally N2) as a result of one or more axillary nodal beds also being involved. In addition trunk lesions, especially those close to the mid-line are one of the least predictable in terms of where they are likely to spread in the neck. Further, while outcome for most melanomas is a function of primary melanoma thickness the exception to this rule is mucosal melanoma where even thin lesions are often lethal.
6. Adjuvant radiation after neck dissection appears to minimise recurrence in the neck but does not appear to impact on survival. A prospective trial would be required to clarify this for certain.

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Diagnosis and Treatment Options for Brain Metastasis of Melanoma

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1. Introduction

Metastasis to the brain is a devastating and common consequence for patients with malignant melanoma. A significant number of patients with melanoma eventually develop brain metastasis at the time of death. Patients are often symptomatic from their lesions and a large percentage of those with neurological deficits eventually die from the brain metastasis. Diagnosis does not typically occur until late in the disease course, which can preclude many treatment options. Additionally, rapid progression of the disease state and worsening health status magnifies the difficulties of treatment. Currently, contrast-enhanced computer tomography (CT) and magnetic resonance imaging (MRI) remain the main diagnostic modalities. Confirmation is usually achieved with surgical biopsy or resection. After diagnosis, treatment options are somewhat limited - surgical management, radiation therapy, and chemotherapy are most commonly used either alone or in combination.

This chapter provides a description of the common presenting symptoms, diagnostic modalities, and treatment options for patients with metastatic melanoma to the brain. This chapter will also discuss emerging technologies which may have notable impacts on the future of disease management. Ultimately, prompt diagnosis and treatment for patients with brain metastases may have important implications for the duration and quality of life of these patients.

2. Epidemiology and demographics

In general, patients with intracranial metastases significantly outnumber those with primary brain tumors. However, there are a small number of population-based epidemiological studies that address the true incidence of intracranial metastases, and studies devoted primarily to intracranial melanoma metastases are even less common [1]. Along with the limitations inherent to most surveys, such as sampling size and variability, there are several other limiting factors. This includes inadequate reporting, difficulty attaining ante-mortem diagnosis, and greater emphasis cancer databases place on the incidence of primary tumors rather than metastasis [2]. As a result, it is quite likely that current population-based epidemiological studies underestimate the true incidence of cancer metastasizing to the brain [2]. A significant amount of the reported data now originates from clinical, neurosurgical, and autopsy series which are subject to their own limitations as well.

However, as methods of diagnosis and treatment continue to improve, a more accurate picture can be portrayed.

Malignant melanoma is one of the most common systemic cancers to metastasize to the central nervous system (CNS). Following lung and breast carcinoma, melanoma historically has the third highest incidence of metastasis to the brain [3]. One recent study has indicated it may now surpass that of breast carcinoma, most likely a result of increasing rates over time [1]. Of cases with metastasis to the brain, melanoma is the primary tumor for about 5 to 21 percent of these patients [4]. CNS involvement or deficits are the first manifestation of melanoma in 9 to 12 percent of patients [5]. For those that carry a diagnosis of melanoma, between 12 to 60 percent can expect to develop metastases to the brain [6, 7]. However, because it only accounts for 5% of metastatic cancers [8], the total number of cases or individuals is often erroneous [1]. Although melanoma is a less common cancer, it has the highest propensity for metastasis to the brain [1, 9]. An estimated 49 to 73 percent of patients who die from melanoma will have developed brain metastases by the time of death and are found on autopsy [10, 11]. It is responsible for the deaths in an estimated 20 to 55 percent of affected patients, and contributes to death in up to 95 percent of all cases [11-13]. Thus, the impact and consequences of metastatic melanoma are quite detrimental in medicine.

Anyone with a diagnosis of melanoma is at risk for developing CNS metastases. Previous studies have tried to elucidate these factors that increase the risk of CNS metastases. Among the demographic aspects intrinsic to patient demographics, only male gender was found to show greater predominance in patients with brain metastases [14]. Of the characteristics of the primary lesion, melanomas appearing wide, thick, or ulcerated or with acral lentiginous or nodular histological findings were more frequently found in patients who developed brain metastases [14]. Also, primary lesions arising from the mucosal surfaces, skin of the head and neck, or skin of the trunk were more frequently found in this group [14, 15]. Patients with involvement of the lymph nodes or visceral organs, especially the lungs or multiple visceral organs, showed an increased likelihood of metastasizing to the brain [14, 16]. These factors are also associated with shortened overall survival time survival times [14, 16]. Interestingly, with the exception of primary lesions of the head and neck region, these factors did not affect survival after a diagnosis of a brain metastasis [14]. Other factors that were evaluated, such as the patient's race, pigmentation of the primary tumor, and pregnancy at the time of melanoma diagnosis, were not significantly correlated with the development of brain metastases [14]. The average age of presentation of patients with brain metastases is 48 to 53 years old, which is similar to that of patients with extracranial metastases [14, 17].

3. Pathophysiology

Metastases to the brain requires a complex series of steps, each mediated by a combination of intricate molecular mechanisms that are not completely understood. Each of these steps typically involves overcoming various physiological barriers including the blood-brain barrier [2]. Similar to other systemic cancers, as the primary melanoma matures, the process of angiogenesis increases the vascular supply to sustain the metabolic needs of the cancer cells and allows the tumor to grow. It progressively invades the surrounding host tissue and eventually spread hematogenously by invading local venules or lymph channels, which drain into the venous circulation [2]. Because venous circulation returns to the right side of the heart, the first capillary beds the circulating tumor cells encounter are typically found in

the lungs. These tumor cells are generally larger than the capillary vessels and may arrest in these pulmonary capillary beds. As a result, patients typically have lung metastases earlier in the time course of melanoma. They may often be identified at the time intracranial metastases are diagnosed. Between about 27 to 68 percent of affected patients may have concurrent lung metastases, which further shortens the survival time [14, 18, 19]. In order to reach arterial circulation and thus the cerebral vasculature, these melanoma cells must reach the left side of the heart either by: (1) metastasizing to the lung and invading the pulmonary venous circulation, (2) traversing the lung capillary bed to the pulmonary venous circulation, or (3) crossing through a patent foramen ovale thus bypassing the pulmonary circulation [2].

When tumor cells reach the left side of the heart and systemic circulation, the most important factors involved in promoting intracranial metastasis are the blood supply and greater preference for brain tissue [2]. The cerebral vasculature receives approximately 15 to 20 percent of the cardiac output in the resting state, which increases the likelihood that circulating tumor cells will reach the brain [2]. It would be expected to receive a proportional amount as well, however the distribution of metastases based on blood flow, or the mechanical hypothesis, does not account for the high propensity of melanoma to metastasize to the brain compared to other cancers [20]. Instead, the seed and soil hypothesis likely contributes to the metastasis and plays an important role in explaining this phenomenon. This hypothesis postulates that certain genetic alterations in the tumor cells (the seed) influences them to show preference for the brain and find its microenvironment a more favorable place (the soil) to support their growth [20]. These alterations may include increased expression of adhesion molecules that show preferential adhesion to brain endothelial cells [21, 22] and increased production of degradative enzymes enabling tumor cells to penetrate the endothelium and the basement membrane [23]. Locally produced growth factors in the brain may also stimulate growth of the metastatic cells [24].

When tumor cells reach the cerebral vasculature, they may arrest in the capillary beds due to their greater size. In order to form metastases, they must extravasate across the microvasculature of the blood-brain barrier into the brain parenchyma [2]. The blood-brain barrier is a continuous, non-fenestrated endothelium composed of tight junctions and protects against the invasion of microorganisms and also the interaction of most drugs, including chemotherapeutic drugs [25]. However, it provides little protection against the invasion of metastatic cells into the brain parenchyma and may even be altered to a leakier barrier in primary tumors and metastases [26]. The cells adhere to and penetrate the basement membrane and astrocytic foot processes, eventually reaching the parenchyma.

In the end, only about 0.1 percent of the initial circulating tumor cells survive the protective mechanisms of the body to form distant metastases [7]. Additionally, metastasis typically occurs relatively late in the disease course for most patients with malignant melanoma. This may be explained by CNS involvement occurring as a result of a late metastatic event from another distant metastatic site, such as the lungs [7]. It may also be possible that metastasis is actually an early event in the disease course, but relatively slow metastatic growth results in delayed neurological effects and delayed detection [7].

4. Pathology

The histopathology of intracranial metastases mimics that of the primary melanoma. Melanoma can metastasize to virtually any portion of the intracranial cavity. The most

common site is the parenchyma, but involvement of any anatomic structure in the CNS can occur, including the dura, leptomeninges, choroid plexus, pituitary, and pineal glands. As with other systemic cancers that metastasize to the brain, the distribution reflects the size and volume of the region and its vasculature. Thus, a significant majority are supratentorial, the most common location being the cerebral hemispheres along the vascular distribution of the border zones (water-shed areas) between the anterior and middle cerebral arteries as well as the middle and posterior cerebral arteries [6, 14]. In total, the parietal lobe is involved in about 26 to 45%, frontal lobe in 21 to 36%, temporal lobe in 19%, occipital lobe in 11%, cerebellum in 7%, and cerebellum in <1%. The spinal cord is rarely involved [6, 14]. About 75 percent of metastases are found in the gray-white junction, where supplying cerebral vessels are slightly constricted, resulting in reduction of blood flow and thus increased risk of tumor cells arrest [6]. Melanoma is also known to have an increased likelihood of developing multiple metastases. Approximately 16 to 61% of patients will have more than one intracranial lesion at the time of diagnosis [14, 17, 18]. Individual lesions are usually relatively small with the largest typically measuring between 1 to 4 cm in diameter, while few are rarely greater than 4 cm. Larger intracranial lesions are noticeably less common and will most often be solitary [6, 14, 18]. Similar to other systemic cancers, the metastases from melanoma tend to expand as roughly spherical masses and establish well-defined interfaces with the surrounding brain parenchyma. Thus, expansion pushes the normal surrounding tissue aside rather than invading it. This contrasts from most primary brain tumors which often show diffusely infiltrated margins [27].

Metastases from melanoma have the highest risk of hemorrhage as compared to other systemic metastases to the brain. Hemorrhage is found on neuroimaging in 27 to 40% of patients with intracranial lesions, while histopathological evaluation has indicated that 62 to 71% of patients have evidence of a prior hemorrhage [6, 18]. The bleeding may be confined to the intracranial lesion itself, extend into the area surrounding it, or expand into an intracerebral hematoma. When multiple metastases are present, simultaneous hemorrhage usually occurs rather than isolated hemorrhage of individual lesions [28]. This most often results in subacute progression which is characteristic of non-hemorrhagic brain metastases. Occasionally, it can cause significant complications such as hematomas and hydrocephalus from obstruction of cerebrospinal fluid flow [18]. Vasogenic edema surrounding brain metastases is also common and can cause similar effects as hemorrhage.

5. Clinical findings

The clinical presentation of melanoma metastases to the brain does not significantly differ from that of other intracranial metastases or primary brain tumors. The presenting signs and symptoms are dependent on the number and location of the lesions as well as the rate of growth. Regardless of etiology, most CNS lesions produce clinical effects either through compression of surrounding neurological tissue or destruction of neurons. For intracranial metastases, the primary mechanism of action is compression from the local mass effect of tumor expansion or secondary effects from raised intracranial pressure or impediment of cerebrospinal fluid circulation. Most patients present with nonfocal complaints secondary to increased intracranial pressure [19]. Common symptoms of increased intracranial pressure include headaches, mental change, somnolence, and nausea and vomiting. Focal or generalized seizures resulting from irritation of neurons are also common in patients with brain metastases [6]. Patients with a single metastasis often present with additional focal

signs and symptoms. This can include such neurological deficits as cranial nerve palsies, visual deficits, hemiparesis, and hemisensory loss[6]. Generally, these nonfocal or focal complaints can present in up to 75 percent of patients with brain metastases, whereas the incidence of seizures in patients may be as high as 50 percent [6, 14, 29]. Although patients with metastases of melanoma to the brain can present with a range of signs and symptoms, there are still a number of patients who may have few or no obvious indications of an underlying pathology[14, 29]. This may be due to insufficient mass effect, however their growth rate compared to primary brain tumors is notably faster. Clinical effects may then be subacute in onset, presenting over weeks rather than months. An acute onset may also occur in such instances as hemorrhagic transformation, dubbed a "tumor TIA". Thus, it is important for physicians to have a high index of suspicion when dealing with patients at risk of developing brain metastases. It is a strong possibility and should be high on the list of differential diagnosis in any patient with a history of melanoma that presents with new neurological signs or symptoms [6].

6. Diagnosis

When there is a high clinical suspicion for metastatic melanoma to the brain based on the history and neurological exam of a patient, neuroimaging is the most important diagnostic modality. Currently, computed tomography (CT) and magnetic resonance imaging (MRI) provide the most beneficial imaging of the CNS. Between these two modalities, MRI with and without gadolinium contrast enhancement is the preferred choice for all systemic metastases. MRI is known to increase the conspicuity of lesions and have increased sensitivity in detecting the presence of additional and smaller metastases to the brain [30, 31]. However, because CT is more readily available in emergent situations, it is often used to image large lesions, hemorrhage, and significant edema, but will be insufficient to definitively rule out intracranial disease [6]. If a single metastasis is found on CT or the scan appears within normal limits, MRI with administration of contrast is warranted because of its improved abilities for detection of lesions [30, 31]. Other radiographic imaging modalities, such as positron emission tomography (PET) with ^{18}F -fluorodeoxyglucose, generally are not useful in the diagnosis or imaging metastatic melanoma of the brain[32]. In the majority of patients, MRI is often the only necessary diagnostic test. However, neuroimaging cannot unequivocally differentiate metastases from other intracranial pathologies. The presence of a lesion on a CT or MRI scan in patient with melanoma or other progressive systemic cancer is not always diagnostic for metastatic spread. The differential diagnosis often includes primary intracranial tumors, cerebral abscess, demyelinating disease, and cerebral infarction or hemorrhage. Each of these etiologies will likely need to be carefully ruled out, however certain characteristics provide strong evidence of metastases. Brain metastases from melanoma frequently appear bright on T1-weighted images and dark on T2-weighted images. This appearance may be attributed to the production of melanin in the tumor [33, 34]. If hemorrhage occurs, the presence of blood breakdown products can alter the T1-weighted or T2 weighted signal[34, 35]. On CT, brain metastases from melanoma are typically slightly hyperdense compared to the surrounding nervous tissue and exhibit moderate contrast enhancement [6]. Increasing the volume of contrast material injected as well as delaying imaging after the injection of intravenous contrast material may improve detection and conspicuity of lesions on CT examination [36]. When the metastases are small, uniform contrast enhancement is typical, but when larger,

peripheral ring enhancement may occur. This ring is usually thicker in comparison to an abscess and more regular than a primary tumor. As noted previously, metastases are usually found at the gray-white junction in the watershed areas of the brain and are typically spherical in shape with more regular margins and a substantial amount of vasogenic edema surrounding a small tumor nidus [6, 14, 27].

After neuroimaging, confirmatory diagnosis can be accomplished through surgical biopsy or, preferably, excision of the entire mass. This allows for definitive differentiation of metastases originating from melanoma versus metastases from possible systemic cancers and other suspected etiologies. If more than one lesion exists, the largest or most symptomatic one should be addressed first for biopsy or resection. Once a brain metastasis has been discovered either through imaging or biopsy, screening for the primary systemic cancer is necessary if one has not already been diagnosed. In contrast, for those patients who have been diagnosed with malignant melanoma but their neurological exam is within normal limits, routine screening of the CNS with neuroimaging rarely identifies metastases and is thus generally not recommended [37].

7. Survival

The prognosis of patients with disseminated melanoma is particularly poor if the CNS is involved. Currently, no reliably curative treatments are available for patients and most therapeutic trials for melanoma exclude patients with brain metastases [38]. For those with documented brain metastases, the overall median survival time is between 3.8 and 5.2 months, a notably shorter survival time in comparison to patients with other sites of distant metastases. The survival percentages are inversely proportional to the length of time after diagnosis [39-43]. Additionally, the median time between the diagnosis of primary melanoma and the diagnosis of metastatic melanoma to brain is about 3.1 to 3.7 years [13, 14, 41]. Among a number of clinical and pathologic factors that have been analyzed, the disease free-interval (DFI) is one of a few that independently predicts survival after diagnosis of AJCC stage IV melanoma. Significant survival benefit occurs when the DFI is greater than 12 months. The anatomical site of the primary melanoma has also been shown to have predictive value. There is an almost fourfold difference between sites associated with highest survival and lowest survival rate. Melanomas with primary metastasis from the skin and lymph nodes have a median survival of about 15 months, whereas those from the brain and liver have a median survival of about 4 months. The third factor that independently predicts survival is the preceding stage of disease before the patient is diagnosed with stage IV melanoma. Patients experience significant survival benefit if they develop stage IV melanoma without progressing through stage III and have a DFI greater than or equal to 72 months, or if they progress through stage III to stage IV and have a DFI greater than or equal to 18 months. Other factors such as gender, age, Breslow depth, Clark level, year of diagnosis, and number of metastatic sites have not shown predictive value [39].

8. Supportive therapy

Several approaches can be taken to provide patients with supportive measures until more definitive treatment can be considered and administered. This primarily involves the medical management of cerebral edema and the resulting increased intracranial pressure, the control of seizures, and the prevention of other associated complications and conditions.

These are generally the most common medical problems in patients with metastasis of melanoma to the brain as well as most other brain tumor. The overall survival in patients with brain metastases treated with only supportive care is approximately 1 to 2 months[14].

8.1 Steroids

The vasogenic edema that is characteristic of metastases is a significant factor in the morbidity of patients. It occurs as a result of BBB break down, allowing sodium and water to leak into and accumulate in the extracellular space of the brain parenchyma[44, 45]. Corticosteroids have been shown to adequately manage this vasogenic edema and can dramatically improve a patient's condition[46, 47]. The beneficial effects are often noticeable within 6 to 24 hours after the first dose and reach maximum effect within 3 to 7 days[48]. Its mechanism of action is quite complex and not completely understood. The antiedema effect may be contributed to stabilization and reduction of the permeability of tumor capillaries through endothelial cell interactions[44, 45]. Corticosteroids are usually indicated in any patient who is symptomatic from the metastatic edema and during the course of definitive treatment with radiation or surgery, but it is typically not necessary in asymptomatic patients with small metastases unless treatment with radiation or surgery is expected[49, 50]. Dexamethasone is administered most commonly because it has minimal mineralocorticoid activity and may have a lower risk of complications compared to other corticosteroids[47]. Long-term use of corticosteroids can result in such significant adverse effects as myopathy, osteoporosis and avascular bone necrosis, diabetes mellitus, cognitive dysfunction, gastrointestinal (GI) hemorrhage, bowel perforation, and opportunistic infections, such as *Pneumocystis jirovecii* pneumonitis and oropharyngeal candidiasis[51]. In instances of significant mass effect or acute decompensation of brain metastases, the onset of effects of corticosteroids is not quick enough and thus the approach to medical management changes[45]. Acute decompensation can be due to a number of causes such as intratumoral hemorrhage, obstructive hydrocephalus, seizures, or hyponatremia. The resulting acutely increased intracranial pressure should be addressed immediately in order to minimize the risk of herniation or worse[47]. This involves stabilization of the BBB, minimization of the vasogenic edema, and emergent intervention with surgical debulking or irradiation. Elevation of the head of the bed, hyperventilation, mannitol, diuretics and are all rapid onset measures that can provide support until steroids take effect and should precede any neuroimaging[49, 52].

8.2 Antiepileptics

Seizures are a common occurrence in patients with brain metastases. The likelihood of seizures is highest in patients with melanoma compared to other cancers including lung and breast carcinoma[51]. Those who present with seizures should generally be treated with antiepileptic drugs (AED)[49]. The use of prophylaxis is often based on individual preference of the treating physician rather than supporting clinical evidence[45]. No significant benefit has been shown with anticonvulsant prophylaxis using phenobarbital, phenytoin, or valproic acid in patients who had no history of seizures[45]. In addition to the lack of efficacy, the risk of potential side effects has been shown to be increased in patients with brain tumors[53]. Overall, almost 25 percent of patients diagnosed with either a primary or metastatic brain tumor and are taking AEDs experience side effects severe enough to warrant a change in or discontinuation of therapy [53]. Thus, it is recommended

that prophylactic anticonvulsants should not be routinely used in patients with newly diagnosed brain tumors[53]. However, because there is a high risk of recurrence, long-term treatment with AEDs is indicated after a patient with melanoma metastases to the brain has suffered their first seizure[51]. Phenytoin has historically been the mainstay of anticonvulsant therapy because it is generally effective and well tolerated. Selection of the particular AEDs to administer requires careful consideration of the treatment the patient is receiving. This is because important interactions can occur with other drugs commonly used in treatment for brain metastases, such as antineoplastic agents and dexamethasone, often from activation of hepatic metabolism through the cytochrome P450 enzyme system[49]. Newer AEDs, including levetiracetam and topiramate, typically do not affect cytochrome P450 and have shown greater reduced seizure frequency and fewer side effects[54].

Anticonvulsant prophylaxis after supratentorial surgery is generally recommended for patients, including those undergoing resection of brain metastases. AEDs have been shown to be beneficial in preventing early seizures postoperatively[45]. However, there is not strong evidence to support the use of long-term treatment to reduce the incidence of late seizures after supratentorial surgery. Thus, in those patients with brain metastases from melanoma who have not had a seizure, the recommended plan for antiepileptic therapy is to gradually taper and discontinue AEDs after the first postoperative week. This is especially appropriate for patients who are medically stable and are experiencing notable side effects from their anticonvulsant medication[53].

8.3 Anticoagulants

Patients with any systemic cancer are known to be in a hypercoagulable state, increasing their risk for deep venous thrombosis (DVTs) and venous thromboembolisms (VTEs). This is especially true for brain metastases for which thromboembolic disease contributes significantly to morbidity and mortality[55]. The risk is often greatest in hemiplegic patients and in the postoperative period since patients are often immobile. In order to prevent DVTs and VTEs from occurring after craniotomy, adequate prophylaxis is often necessary. However, this can be difficult due to the possibility of intratumoral hemorrhage and intracranial bleeding with anticoagulation therapy. Current methods of prophylaxis include mechanical and/or pharmacological interventions, however no optimal one has been identified and current recommendations remain controversial[56]. Unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH) are the main pharmacological anticoagulants which inhibit formation of thrombi. Mechanical methods attempt to minimize venous stasis and enhance fibrinolysis. This includes early ambulation, compression stocking, intermittent external pneumatic compression devices, and electrical calf muscle stimulation. In general, both approaches are effective in preventing DVTs and VTEs[57], but heparin may be more effective although at a greater risk of intracranial hemorrhage[45]. Mechanical prophylaxis with concomitant anticoagulation therapy during the postoperative period is not only safe but also protects patients more so than either approach does alone[57].

In patients that have developed a DVT, treatment is necessary to prevent a pulmonary embolism (PE), restore lower limb circulation, and resolve other associated problems. Pharmacological treatment with UFH and LMWH are the mainstays of therapy, with LMWH showing better outcomes including fewer bleeding complications. Patients who have strict contraindications against anticoagulation can be treated with placement of an

inferior vena cava (IVC) filter[49]. However, these should generally not be the first line of treatment because it has a higher complication rate and are less effective in prevent PE in comparison to anticoagulation. One retrospective study of IVC filter complications occurring in patients with brain tumors and DVT found that there was a complication rate of 62 percent and PE still occurred in 12 percent of cases despite proper placement of the IVC filter[58]. Thus, IVC filters should be reserved for patients that have had recent craniotomy, are at increased risk for intracranial hemorrhage, are poorly compliant with medications, or will have prolonged thrombocytopenia from chemotherapy.

9. Definitive treatment

After receiving supportive measures, patients diagnosed with melanoma that has metastasized to the central nervous system must be evaluated for the possibility and type of definitive treatment. Current options available to physicians include whole brain radiation therapy (WBRT), stereotactic surgery, conventional surgical resection, or chemotherapy. These can frequently be used alone or in combination with one other. However, for the majority of patients, these are largely palliative measures. Determining the optimal modality is dependent on a number of factors including the size, number, location, and sensitivity of the lesion, the overall status of the malignant melanoma, the neurological status as measured by the Karnofsky Performance Scale (KPS), general condition of the patient, and the preferences of the patient and his or her family. It can thus be difficult to decide on a course of treatment given the number of issues that need to be considered.

9.1 Whole brain radiation therapy

The first use of external beam WBRT for treatment of brain metastases was reported in 1954 by Chao et al[59] and again later in 1961 by Chu et al[60]. It has since become an important treatment modality for brain metastases. One of the fundamental benefits of WBRT is that it is a noninvasive means in which to treat the entire brain and provide palliation of symptoms. Thus, it allows for relatively simple targeting of any and all lesions in the brain with radiation including microscopic ones, micrometastases, which are not detected on neuroimaging. This has been demonstrated in studies which showed that prophylactic and postoperative irradiation of the brain decreases subsequent development of intracranial metastases. This effect is most likely due to elimination of micrometastases that were present at the time[61, 62]. External WBRT is thus advantageous and typically considered the mainstay of treatment for most patients with multiple metastatic deposits from melanoma in the brain[38, 63, 64]. More localized treatment modalities would be less beneficial in such situations because it would require targeting of each lesion individually. However, solitary metastases that are too large for either surgical resection or stereotactic surgery or those that impinge on sensitive areas of the brain are often treated with WBRT[63, 65].

The broad application of radiation to the brain can also be an important disadvantage. This is because it not only affects the malignant tissue, but the normal tissue is also exposed to the harmful effects of ionizing radiation. Side effects are typically dependent on the total dosage, dosing interval, and fraction size. Acute side effects of external WBRT include memory loss, fatigue, headaches, temporary hair loss, scalp rash or desquamation, hyperpigmentation, otitis media, and cerebral edema[63, 66]. Somnolence syndrome is a set of symptoms, often seen in children, involving lethargy, anorexia, and irritability that

present 1 to 4 months after treatment. There are generally no focal neurological deficits with this syndrome[66-68]. If the patient survives long-term, late side effects may occur which include cerebral edema, atrophy, focal radiation necrosis, white matter demyelination, leukoencephalopathy, endocrinopathy, and progressive cognitive dysfunction[69-71]. A recent randomized controlled trial found that patients suffered significantly greater decline in learning and memory functions after receiving WBRT compared to patients only receiving stereotactic radiosurgery[72]. These consequences should be considered if the patient has the potential to survive for a prolonged amount of time following radiation treatment. Another issue that is important when considering external WBRT for treatment of metastatic melanoma to the brain is the significant resistance melanoma has to this mode of radiation therapy. Of all the primary tumor types, malignant melanoma is considered to be one of the most radioresistant to WBRT[73]. Larger fractions may be necessary in order to achieve desired effect, increasing the likelihood for negative side effects[74]. However, because there are few other effective modalities for treatment of multiple metastases in the brain, it is still commonly used in these patients.

The Radiation Therapy Oncology Group (RTOG) conducted several extensive phase III randomized trials to evaluate the efficacy of various treatment schedules. The results of which indicated that 30 Gy administered in 10 fractions of 3 Gy over a period of 2 weeks results in palliative results and survival time equivalent to more protracted and higher-dose schedules[75, 76]. This has since become the most commonly used external WBRT schedule in the United States for brain metastases in general. Although this is often inadequate for long-term tumor control except in the most radiosensitive histologies, it allows for minimization of toxicity and negative side effects of irradiation. The median survival after administration of WBRT to patients with brain metastases is typically improved to about 3 to 6 months but is dependent on the number of lesions, the radiosensitivity of the metastases, and the status of the underlying cancer. Despite its known general resistance to radiation therapy, studies have shown local tumor response of melanoma metastases to the brain after administration of WBRT[77, 78]. Many fractionation schemes have been devised with larger doses per fraction in an attempt to enhance this tumor response. However, a review of several retrospective series has revealed that no scheme is better than the current standard of 30 Gy in 10 fractions[38]. Improved clinical outcomes may occur after WBRT, showing mildly increased median survival times to about 2.0 to 6.1 months[79-82]. When patients are stratified according to the RTOG recursive partitioning analysis (RPA), the effect of WBRT can be better extrapolated. The RPA separates patients into three prognostic groups according to their KPS, extracranial disease, and patient age. Those in RPA class 1 frequently have the best prognosis due to younger age (<65 years) and higher KPS scores (>70) whereas RPA class 3 often have the worst prognosis due to lower KPS scores (<70). The survival times after WBRT expectedly correlate with RPA class. Those with brain metastases originating from melanoma had median survival times of about 7.1 to 10.5 for patients in class 1, 4.2 to 5.9 for patients in class 2, and 1.8 to 2.3 for patients in class 3[82, 83]. Despite only having minimal effects on the survival time, using external WBRT and supportive management in patients with metastatic melanoma of the brain has demonstrated palliation of symptoms[79]. Symptomatic improvement is an important effect that can enhance the quality of life given the bleak prognosis despite all treatment modalities. Patients often have improvement of headaches, weakness, and mental status. There are some who question its ability to reverse neurological symptoms and suggest omitting WBRT melanoma metastases are fewer than four[72]. However, WBRT still

currently remains the mainstay of treatment for patients with multiple brain metastases but is not typically first-line in solitary metastases [65]. It is a viable adjuvant therapy in addition to serving as an option for primary therapy. In cases of single brain metastases, surgical resection or stereotactic radiosurgery are usually the preferable option for primary therapy unless both are contraindicated. Patients that additionally have advanced systemic disease or conditions that preclude surgery or radiosurgery in addition to a solitary lesion would likely be better suited for WBRT.

9.2 Surgical resection

Surgical resection was first reported for use in the treatment of brain metastases in 1926 by Grant[84]. As noted earlier, metastatic brain tumors characteristically form well-circumscribed and rounded masses at the junction of the gray and white matter. This renders them highly amenable to surgical resection. Additionally, with modern neurosurgical techniques and the available new technologies such as functional mapping, intraoperative ultrasonography, and computer assisted stereotaxy, surgical resection can be accomplished with increased precision and control. It has become a mainstay of treatment despite the development of newer methods including WBRT or stereotactic radiosurgery. This is because it offers several advantages over both. Surgery (**Figure 1**) provides immediate palliative action and relief of symptoms with removal of the lesion, which decreases the intracranial pressure, alleviates compression and mass effect on the surrounding parenchyma, prevents or stops hemorrhage and edema into the intracranial space, and restores CSF flow if obstruction has occurred. No other treatment modality can provide this immediate effect which is critical in emergent situations such as impending herniation or posterior fossa tumors. Removal of the tumor with surgical resection additionally provides diagnostic advantages. It is the only modality that allows for physical extraction of the mass in order to determine a histological and pathological diagnosis. This is important in patients in which the etiology of the lesion is uncertain or the primary cancer has not been identified. Studies have demonstrated that up to approximately 11 percent of suspected cranial metastases are actually found to be nonmetastatic lesions, such as cerebral abscesses or primary tumors, on pathological evaluation[85]. Surgical resection also avoids some of the prominent drawbacks of WBRT, most notably the resistance of melanoma metastases to radiation therapy and the negative effects of diffuse radiation on normal neurological tissue. Instead, it circumvents the use of radiation and surgically localizes the area of the metastases, minimizing damage to the rest of the unaffected brain parenchyma and avoiding the acute and long-term side effects depicted in WBRT. Surgical resection is thus most advantageous for solitary or a limited number of metastases to brain where diffuse involvement does not occur. However, there is still significant morbidity and mortality associated with surgical resection given its invasive nature. These risks continue to gradually improve with the advancement of available procedures and tools, as was seen with the utilization of CT and MRI neuroimaging. Several recent studies have shown the risk of mortality to be between 0 to 14.2 percent during the postoperative period [14, 18, 86, 87] which is compared to earlier reports of mortality in up to 22 percent of patients[87]. Given the limited survival time of patients harboring metastatic melanoma of the brain, postoperative neurological deficits and prolonged recovery times are best avoided if possible. Patient selection is important in minimizing poor outcomes and maximizing the response to treatment. In general, surgical resection is most appropriate for patients with a

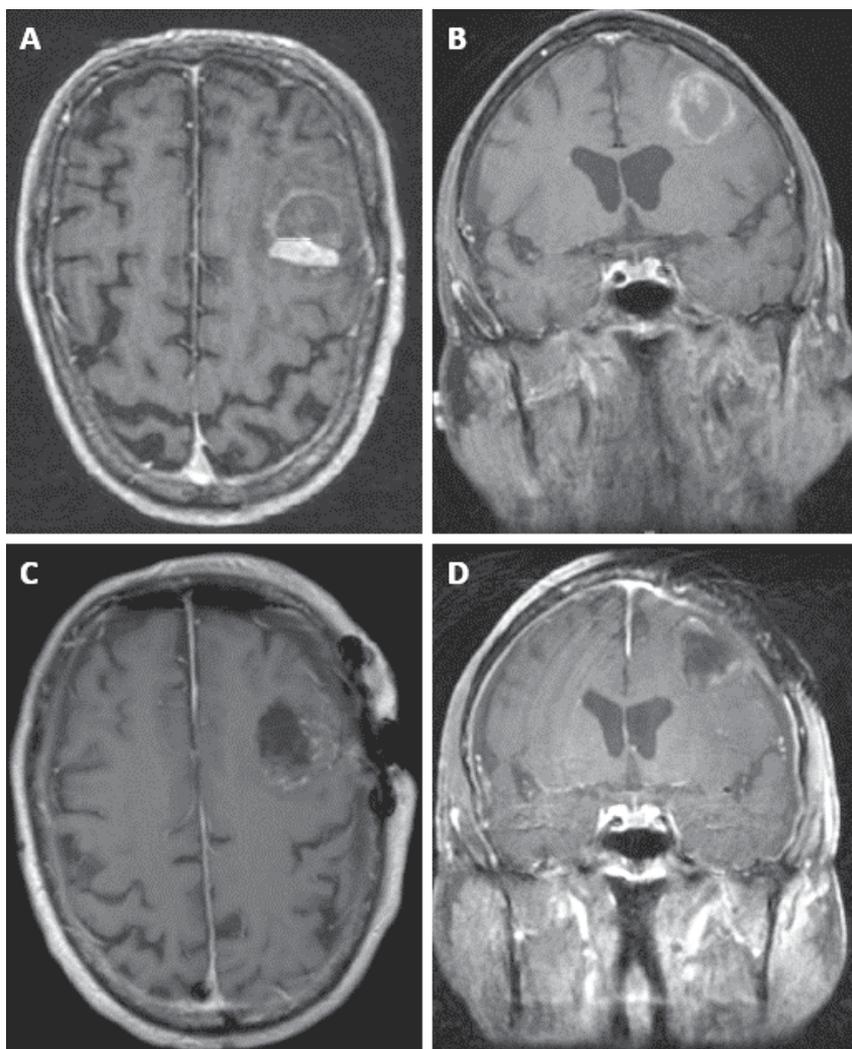


Fig. 1. Representative patient with intracranial metastatic melanoma who underwent surgical resection. **A**, pre-operative contrast-enhanced axial and **B**, coronal T1 magnetic resonance image (MRI) demonstrating left frontal metastasis with intratumoral hemorrhage. **C**, post-operative contrast-enhanced axial and **D**, coronal T1 MRI demonstrating gross total resection of the metastasis.

single brain metastasis, limited systemic disease, and favorable KPS score (>70). Metastases greater than 3 cm in diameter should be preferentially treated with surgery because large tumors typically have minimal response to the other modalities[88]. Selection of patients with multiple metastases to the brain remains somewhat controversial. Several retrospective studies have examined the efficacy of surgical resection alone in the treatment of brain

metastases from melanoma. It is clear that surgical resection of a single melanoma metastases greater extends the survival time in comparison WBRT alone or supportive therapy alone. In these studies, the median survival time after surgery varies between 5 and 22 months with the more recent ones showing a median survival time between 8 and 16 months[87-89]. In contrast to solitary metastasis, the role of surgical intervention in cases of multiple brain metastases remains controversial due to lack of randomized control trials[63]. Previously, this was typically a contraindication, but one retrospective study challenged this notion. Bindel et al[90] compared patients with single and multiple brain metastases that were treated with surgical resection. Patients that underwent partial resection of multiple metastases, complete resection of multiple metastases, and resection of a single metastasis all had similar rates of surgical morbidity and mortality of approximately 8 to 9 percent and 0 to 4 percent, respectively. However, those patients that underwent only a partial resection demonstrated a median survival time of about 6 months, whereas those that were treated with complete resection were found to have a longer median survival time of about 14 months. This provided evidence that total surgical resection of multiple metastases was comparable to resection of a solitary metastasis in efficacy. Several ensuing studies showed similar support for the use of surgical resection in the presence of multiple metastases[91, 92]. Thus, surgical resection of multiple metastases can be supported, although the general recommendation limits the number to less than 3 lesions in patients that have limited or controlled systemic disease.

A regimen that has been frequently discussed in the treatment of brain metastases is the combination of surgery plus external WBRT. Several studies have been conducted to compare the value of surgical resection followed by WBRT to that of either modality alone. Patchell et al[85] and Vecht et al[93] both demonstrated that those patients who were treated with the combination regimen had longer median survival times than those patients who received only WBRT. In contrast, Mintz et al[94] found that there was no survival advantage or improved quality of life with the administration of WBRT after surgery. This finding may be due to the overall lower patient performance status and greater prevalence of patients with more severe extracranial disease in their study. Patchell et al[85] actually demonstrated that patients with active systemic disease had a poorer prognosis when treated with surgical resection and WBRT. Studies comparing surgery alone with the adjunctive WBRT have also shown mixed results. Dosoretz et al[95] found that WBRT at a total dose of 30 Gy showed no survival advantages after surgical resection of a solitary metastasis. DeAngelis et al[70] and Hagen et al[96] also reported no effect on survival time, but noted WBRT postoperatively may reduce the risk of recurrence and thus recommend its use in patients after surgical resection for a single metastasis. Patchell et al has also further investigated the advantages of this combination regimen. In this prospective trial, patients were randomized to treatment with external WBRT or observation after surgery for a solitary brain metastasis. The results showed that patients who received the WBRT postoperatively had improved tumor control as seen by lower recurrence of metastases anywhere in the brain as well as at the site of resection. As with DeAngelis et al[70] and Hagen et al[96], this trial did not demonstrate prolonged survival time in patients receiving the adjunctive WBRT and did not slow the functional decline of patients, as measured by their KPS scores. Other studies have specifically addressed its use in melanoma metastases and have shown similar nonsignificant affect on prolonging survival times.

9.3 Stereotactic radiosurgery

Stereotactic surgery (SRS), also known as Gamma Knife, was first introduced in 1951 by Leksell[97]. Since then, it has developed into a sophisticated system that has the ability to accurately target an intracranial lesion and administer focal, collimated beams of ionizing radiation produced from a linear accelerator (linacs) or cobalt-60 sources. The radiation dose is administered in a single fraction via numerous crossfiring of beams of radiation that converge onto the targeted site. The crossfiring of these beams from numerous directions allows for rapid radiation falloff in the surrounding tissue and thus minimize extraneous exposure. The advantage of SRS lies in its ability to administer localized treatment while sparing the rest of the normal brain parenchyma from the diffuse irradiation that occurs in WBRT. Moreover, the mechanism of action of SRS is thought to be different than WBRT and may instead affect the tumor vasculature, which would increase its effectiveness against the typically radioresistant cancers including malignant melanoma[98]. The advantage of SRS (**Figure 2**) over surgical resection is attributed to its ability to reach small, deep metastases in the brain without significant disruption to the rest of the brain parenchyma. Thus, it avoids the prolonged recovery time, increased length of hospital stay, and high costs that occur with an invasive surgery and instead only requires the administration of a single-fraction of radiation. Although it generally avoids these immediate side effects of surgical resection, delayed complications from SRS can occur which typically resemble other radiation-induced side effects such as radiation necrosis. This occurs in less than 10 percent of patients and is dependent on the volume treated and dose administered.

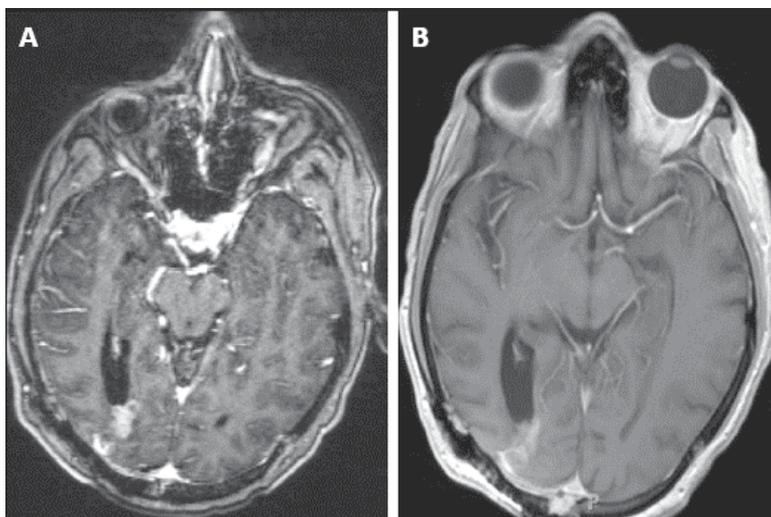


Fig. 2. Representative patient with intracranial metastatic melanoma who underwent stereotactic radiosurgery. **A**, pre-radiation contrast-enhanced axial T1 magnetic resonance image (MRI) demonstrating right occipital lobe metastatic melanoma. **B**, post-radiation contrast-enhanced axial T1 MRI.

There are a few disadvantages of SRS in comparison to the other treatment modalities. In comparison to surgery, its minimally invasive approach cannot provide immediate

treatment effects which are often important in critical or life-threatening situations such as impending herniation. The inability to remove metastases also prevents a definitive histological diagnosis, and given the chance a suspected cranial metastases may actually be a nonmetastatic lesions, pathological diagnosis can be of great importance[85]. Also, increased risks in treating large metastases have hindered its use for those tumors that are larger than 3 cm in diameter. This is due to the limited conformity that can be achieved for large tumors, resulting in decreased response with increasing tumor size and increased radiation doses to the surrounding brain parenchyma[99]. Mehta et al[100] noted that tumors less than 2 cm³ in volume showed a total response rate of 78 percent whereas as tumors greater than 10 cm³ in volume demonstrated a total response rate less than 50 percent. Thus, SRS is typically recommended for patients with lesions less than 3 cm on neuroimaging, patients with lesions that are surgically inaccessible, patients that are asymptomatic, or patients that cannot tolerate surgery.

Multiple studies examining the efficacy of SRS have shown generally positive results. For all types of metastatic histologies, the local control rate after treatment with SRS ranges between 85 and 90 percent at one year[101-103]. For metastatic melanoma to the brain, the local control rate was shown to be approximately 90 to 97 percent[103-105]. Clinically, stabilization or improvement of neurological symptoms after treatment was noted in about 78 to 100 percent of patients[104, 106]. Patients also demonstrated a median survival time comparable to that of surgical resection which was found to be about 5 to 14 months[106-110]. However, as with the other treatment modalities, patient factors and selection affects outcome. Those with minimal extracranial disease typically fared better than their counterparts more extracranial disease. This is also true for patients with overall better performance status, as measured by the KPS, compared to those with lower performance status. Patients with solitary brain lesions additionally demonstrated longer survival times than those patients with multiple brain lesions. However, an increased number of metastases should not be a reason to withhold SRS treatment[106-110].

Combination therapy has been studied and utilized in the treatment of metastatic melanoma to the brain. A landmark RTOG-sponsored study[111] compared the treatment of brain metastases using WBRT alone and SRS plus WBRT. Patients were first stratified according to the number of brain metastases and the extent of their extracranial disease and then randomized to treatment with WBRT only and SRS plus WBRT. There was a significant survival advantage for those harboring a solitary lesion treated with the combination therapy. There was no survival advantage for patients with multiple metastases treated with the combination approach. However, patients treated with WBRT and SRS were more likely to have improved or stable performance status and also decreased need for steroid use after therapy. There was no difference in mental status change and no increase in toxicity with the SRS and WBRT administration. The authors thus concluded that the combination approach of SRS plus WBRT should be the standard treatment for patients with a single unresectable brain metastasis. For patients with two to three brain metastases, despite the survival advantage the combination treatment should be considered because it results in improvement in the overall performance status.

To date, there are no prospective, randomized control trials comparing SRS with surgical resection. However, a few retrospective studies have provided insight into a comparison between the two treatment modalities. Auchter et al[112] designed a multi-institutional

retrospective analysis comparing the outcome of patients treated with surgery and SRS and found no difference in the overall survival, functional independence, or recurrence rate. O'Neill et al[113] conducted a similar comparison and found no difference in median survival time after treatment with either approach. However, there was a significant difference in local tumor control, with no recurrence occurring after administration of SRS and about 58 percent recurrence after surgical resection. Bindel et al[114] conducted a smaller study in which patients undergoing SRS or surgical resection were matched on the basis of age, sex, primary tumor histology, extent of systemic disease, pretreatment KPS score, time to diagnosis of brain metastases, and number of brain metastases. The results demonstrated an overall survival advantage for surgical resection with a median survival time of 16.4 months compared to 7.5 months in the SRS treated group. Surgical resection also showed superiority to SRS in terms of local recurrence and morbidity. Thus, they favored the use of surgery over SRS for the treatment of solitary brain metastases. Cho et al[115] conducted a more encompassing study analyzing the treatment of solitary brain metastases with WBRT only, surgery plus WBRT, or SRS plus WBRT. The results demonstrated that the actuarial survival time was the same for the combination surgery group and the SRS surgery group, and both had longer survival times than patients receiving WBRT alone. Cho et al thus concluded that SRS is a reasonable and potentially more attractive alternative than surgical resection for single brain metastases. There are still several ongoing trials that include SRS in the treatment plan for brain metastases.

9.4 Chemotherapy

The use of chemotherapy for the treatment of extracranial melanoma has generally shown a poor response. Thus, it is not surprising that the response of melanoma metastases to the brain is also poor. The effect of cisplatin and etoposide has been shown to have a 0 percent response rate while other common metastatic cancers such as NSCLC and breast carcinoma have shown up to a 39 percent response. A significant obstacle for chemotherapeutic action on intracranial metastases is the BBB which limits the passage of large molecules into the brain parenchyma. Even after penetrating the BBB, some agents are rapidly eliminated and only have a transient effect. Attempts have been made to identify agents that can adequately cross the BBB to have tumoricidal effects. Fotemustine, a nitrosurea with high penetrations, has been shown to have response rates ranging from 12 to 25 percent in phase II European trials but is not available in the United States[116, 117]. Temozolamide, a dacarbazine analogue with high penetration, was approved for use by the FDA for use in primary brain tumors and was found to have a modest response rate of 7 percent for metastatic melanoma[118]. Combination chemotherapy has also been explored for increased effectiveness. A combination regimen of temozolomide and thalidomide, an antiangiogenic agent, has been explored because of its action against the vascularization of the tumor. Although the response was slightly better, this was at the expense of increased toxicity. A combination of chemotherapy and external WBRT is also being actively explored. Mornex et al[119] compared fotemustine alone versus fotemustine and WBRT and found that the response rates and survival times were not significantly different. Similarly, a phase III trial comparing WBRT alone and temozolomide plus WBRT demonstrated improved response rate but no prolonged survival time[120]. In general, current evidence does not support routinely administering chemotherapeutic agents for the treatment of cerebral metastases from melanoma.

9.5 Immunotherapy

Melanoma is a highly immunogenic tumor and treatment with immunotherapy has been attempted to halt the metastatic process. Predominantly from case studies, the overall response has not been optimal. Traditionally, the diagnosis of brain metastases has been an exclusionary criterion for receiving immunotherapy in patients with melanoma. However, biological response modifiers (BRMs) and cellular immunotherapy have been able to induce infrequent responses from brain metastases. One case report noted the near complete response in a patient with brain metastases after a treatment regimen of interleukin-2 (IL-2), interferon (IFN), and 5-fluorouracil[121]. Anecdotal cases of partial or complete responses have also been reported after ipilimumab therapy for metastatic melanoma to the brain which was treated earlier by surgery or SRS[122]. Another case report identified a patient with brain metastasis refractory to IL-2 and chemotherapy was responsive to lymphodepletion followed by infusion with autologous MART-reactive tumor infiltrating lymphocytes (TILs) and high doses of IL-2[123]. Hong et al[124] investigated the response rate and response duration of melanoma brain metastases to adoptive cell therapy (ACT) with autologous antitumor lymphocytes plus IL-2 following a lymphodepleting preparative regimen. They found that greater than 50 percent of patients had complete or partial response to the treatment regimen with rare occurrence of negative side effects. Majer et al[125] conducted a study of 70 patients with or without brain metastases treated with temozolomide or DTIC plus the BRMs IL-2 and IFN- α 2B. They demonstrated that patients with brain metastases who were treated previously with SRS had a prolonged median survival time of 15.8 months versus 11.1 months in patients without brain involvement. Overall, there have been limited studies and trials into the use of immunotherapy and additional investigation into its efficacy is needed.

10. Conclusions

Metastasis to the brain is a devastating and common consequence for patients with malignant melanoma. A significant number of patients with melanoma eventually develop brain metastasis at the time of death. Current treatment options typically include surgery and radiation therapy for brain metastases but the number of options is increasing. Prolonged survival depends on prompt diagnosis and treatment for patients harboring these lesions.

11. References

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Part 3

Targeted Chemotherapy and Immunotherapy

Chemotherapy

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1. Introduction

The number of melanoma cases worldwide is increasing faster than any other cancer. Although early detection, appropriate surgery, and adjuvant therapy have improved outcomes, the prognosis of metastatic melanoma remains very poor. Advanced melanoma is still associated with an extremely poor median survival, ranging from 2 to 8 months, with only 5% surviving more than 5 years and remains one of the most treatment-refractory malignancies. Many agents have been investigated for antitumor activity in melanoma but the current treatment options for patients with metastatic disease are limited and non-curative in the majority of cases (Mouawad et al, 2010).

The treatment of a patient with metastatic melanoma depends on multiple factors including the overall condition and age of the patient, the sites and number of metastases, pace of the disease, and the patient's wishes for treatment. Currently, the goals of treatment are directed toward palliation of symptoms, particularly if the improvement in the symptoms related to the disease exceeds the side effects associated with the therapy (Green & Schuchter, 1998).

In advanced melanoma, single agent chemotherapy, combination chemotherapy, biochemotherapy (chemoimmunotherapy), targeted therapy, Toll-like receptor agonists and antiangiogenic therapies have been used (Bhatia et al., 2009; Chowdhury, 1999; Cohen & Falkson, 1998; Jilaveanu et al., 2009; Lutzky, 2010; O'Day et al, 2002; Tarhini & Agarwala, 2006; Treisman & Garlie, 2010).

In this chapter, classical chemotherapeutic agents, regimens and new chemotherapeutics, such as targeted therapies for melanoma treatment have been reviewed.

2. Chemotherapy

Melanoma is considered a chemotherapy-resistant disease, and systemic chemotherapy has failed to significantly improve the survival of patients with nonresectable metastatic melanoma. The disease frequently becomes refractory to the agents even after initial responses are observed. Despite the lack of curative effect for the patient with advanced metastatic disease, chemotherapy continues to play a role in palliation of the disease. Although many agents have been used for melanoma treatment, single agent chemotherapy has generally been considered ineffective. Despite the poor overall outcome with these agents, they are still in common use in the clinic (Treisman & Garlie, 2010).

For stage IV melanoma, palliative systemic chemotherapy is the mainstay of treatment and is associated with median survival durations with combination regimens of 6-9 months and

5-year survival rates of approximately 6%, which are not influenced significantly by any therapy yet tested in rigorous multicentre cooperative group trials (Eggermont & Kirkwood, 2004).

2.1 Single agent chemotherapy

A large number of clinical trials have tested different single drugs like alkylating agents, nitrosureas, vinca alkaloids, platinum drugs, taxanes, topoisomerase inhibitors and anthracyclines, but few have shown an objective response rate (<20%) or an increase in progression-free and overall survival rates (Mouawad et al, 2010).

2.1.1 Alkylating agents

Dacarbazine (DTIC) is the first US Food and Drug Administration (FDA) approved chemotherapeutic agent for the treatment of metastatic melanoma (Green & Schuchter, 1998; Mouawad et al, 2010). The response rates with dacarbazine were 15-25%, with median response durations of 5-6 months, but less than 5% of complete responses. Long-term follow-up of patients treated with DTIC alone shows that less than 2% of the patients could survive for 6 years (Mouawad et al, 2010, Sasse et al., 2009). DTIC is a prodrug of the alkylating agent 5-(3-methyltriazen-1-yl) imidazole-4-carboximide (MTIC). The drug is generally well tolerated, with nausea as its major side effect, which can be controlled with antiemetic therapy (Treisman & Garlie, 2010). Doses and schedules of DTIC vary widely, with no data suggest that response rates are influenced by these variables. The most commonly used regimen is 850-1000 mg/m² intravenously, on day 1 only, repeated every 3 weeks (Green & Schuchter, 1998). It can be applied 200 mg/m² intravenously daily for 5 days every 3 weeks (Jilaveanu et al., 2009). It is more effective for subcutaneous, lymph node, and pulmonary metastases (Jilaveanu et al., 2009), but it is ineffective in brain metastases (Marsden et al., 2010). DTIC has been used as a standard for comparing the efficacy of new regimens (Coit et al., 2011; Marsden et al., 2010).

Temozolomide (TMZ), an imidazotetrazine derivative of dacarbazine, is another cytotoxic alkylating agent and has the same active metabolite as dacarbazine. It is usually used to treat solid tumors, such as brain tumors, due to its ability to cross the blood-brain barrier, and might therefore constitute an alternative to DTIC in treating brain metastases, for which dacarbazine is ineffective (Jilaveanu et al., 2009; Treisman & Garlie, 2010).

TMZ was shown to have an objective response rate of 21% (12 of 56 patients) in a phase II study, with a median survival time of 5.5 months (Treisman & Garlie, 2010). Temozolomide was equivalent to dacarbazine in treating metastatic melanoma in a large randomized trial of melanoma patients with incipient metastatic disease. It was administered orally to one group at a dose of 200 mg/m² for 5 days every 4 weeks and compared with dacarbazine administered iv at 250 mg/m² for 5 days every 3 weeks. The median overall survival was 7.7 months for patients treated with TMZ and 6.4 for those treated with DTIC. The 6-month overall survival rate was 61% and 51%, respectively; the overall survival was not statistically significant. The median progression-free survival was significantly longer in patients treated with temozolomide (1.9 months versus 1.5 months). No big differences were observed in toxicity between the two drugs (Jilaveanu et al., 2009). The results of another multicentre phase III trial that randomized 859 patients to receive DTIC vs an extended dosing schedule of TMZ (150 mg/m²/d on 7 consecutive days every 14 days) were recently reported. The investigators found no significant differences between DTIC and TMZ in objective response rate, progression-free survival, or overall survival (Bhatia et al., 2009).

Although temozolomide administered as a single agent might have some advantages in a select group of patients, it has not been FDA-approved for advanced stage melanoma; however, it is widely used in the United States. Temozolomide and dacarbazine are being studied in combination with other therapies (Jilaveanu et al., 2009). The addition of IFN to TMZ resulted in higher response rates; however, survival was similar for both treatments and the combination was associated with higher toxicity. TMZ has shown promising results in the treatment of brain metastases from melanoma and may be a reasonable option if surgery or radiation is not appropriate (Quirt et al., 2007).

In a randomized phase III study, Middleton et al. compared dacarbazine and temozolomide in 305 patients with advanced melanoma. They found that median survival time was 7.7 months for patients treated with temozolomide and 6.4 months for those treated with DTIC. Median progression-free survival was significantly longer in the temozolomide-treated group (1.9 months) than in the DTIC-treated group (1.5 months) ($p=0.012$). No major difference in drug safety was observed. Temozolomide therapy improved health-related quality of life; more patients showed improvement or maintenance of physical functioning at week 12. They concluded that temozolomide demonstrated efficacy equal to that of DTIC and was an oral alternative for patients with advanced metastatic melanoma (Middleton et al., 2000).

2.1.2 Antimicrotubular agents

Microtubular toxins and microtubular disassembly inhibitors have both been used in patients with metastatic melanoma. The vinca alkaloids especially vinblastine and vindesine, based on their modest activities and limited toxicities, have primarily been used in combination therapy (Mouawad et al., 2010; Treisman & Garlie, 2010). They are not effective as monotherapy. Vinflunine ditartrate and vinorelbine are the other vinca alkaloid agents that have been used in phase II trials (Jilaveanu et al., 2009).

Paclitaxel and docetaxel, taxanes, are microtubule disassembly inhibitors with antitumor activity in a variety of neoplastic diseases. Paclitaxel has been evaluated in several phase I and II studies, and has demonstrated an approximately 12% to 16% response rate in previously untreated patients. Paclitaxel is commonly used in combination with carboplatin in other malignancies, and was similarly tested in melanoma (Treisman & Garlie, 2010). Weekly administration of paclitaxel at a dose of 80 to 100 mg/m² (on days 1, 8, and 15 every 4 weeks) is well tolerated by most patients. Alternatively, a higher dose can be administered once every 3 to 4 weeks (Bhatia et al., 2009). A phase II docetaxel study showed a 12.5% response rate in melanoma with one of the patients having a durable complete response, and it still being actively studied in other combinations, with some benefit (Treisman & Garlie, 2010). Several studies have evaluated docetaxel, at a dose of 100 mg/m² administered intravenously over 1 hour every 21 days, with response rates ranging from 15% to 19% (Green & Schuchter, 1998).

Associated toxicities include fatigue, alopecia, myelosuppression, neuropathy, myalgias, and hypersensitivity reactions (Bhatia et al., 2009).

2.1.3 Platinum analogs

Cisplatin and carboplatin have modest activity in patients with metastatic melanoma. Single agent cisplatin given at conventional doses yields a response rate of less than 10%. However, a phase II study that used a higher dose (150 mg/m²) of cisplatin in combination with

amifostine reported an objective response rate of 53%, although the responses were shortlived. A response rate of 19% was observed with carboplatin in a phase II study in chemotherapy-naïve patients with metastatic melanoma. Carboplatin has also been used in combination with paclitaxel in previously treated patients (Bhatia et al., 2009).

2.1.4 Nitrosoureas

The nitrosoureas are a group of alkylating agents that act by cross-linking DNA (Treisman & Garlie, 2010). Carmustine (BCNU), lomustine (CCNU) and fotemustine have single-agent activity comparable to dacarbazine, although they cause more myelosuppression and alopecia. A chloroethyl nitrosourea, fotemustine rapidly crosses the blood-brain barrier and has been found to have encouraging activity in patients with brain metastases. When compared to dacarbazine in a phase III trial involving 229 patients with metastatic melanoma, fotemustine was associated with a higher objective response rate (15% vs 7%, respectively) and a trend toward improved survival (7.3 vs 5.6 months, respectively). In patients without brain metastases at inclusion, the median time to development of brain metastases was 22.7 months in the fotemustine arm vs 7.2 months in the dacarbazine arm. Fotemustine has not been approved by the FDA but is available in Europe (Bhatia et al., 2009).

In clinical practice, these agents have a limited role as single agents, but have been used in combination chemotherapy (Treisman & Garlie, 2010).

2.1.5 Tamoxifen

The identification of estrogen receptors in melanoma led to initial trials of hormonal therapy for the diseases, and tamoxifen, an estrogen receptor antagonist, might be considered one of the first targeted agents used for the therapy for melanoma. Tamoxifen was initially used as a single agent and then in combination with various chemotherapeutic regimens. Although initial studies suggested a benefit for tamoxifen as a single agent in the treatment of metastatic melanoma, subsequent studies showed a response rate of only 5%. In addition, evaluation of samples using immunostaining failed to demonstrate estrogen receptors. Tamoxifen could have several other effects including effects on angiogenesis, synergic effects with chemotherapy, and reversal of multidrug resistance. More recently, preclinical studies have shown that tamoxifen potentiates the cytotoxic action of chemotherapeutic agents, specifically DTIC and cisplatin (Green & Schuchter, 1998). Several randomized clinical trials have been conducted to assess the therapeutic benefit of tamoxifen in combination chemotherapy regimens. Cocconi et al of the Group of Italian Investigators For Cancer Research (GOIRC) compared DTIC alone to DTIC plus tamoxifen in a study of 117 patients. They found a statistically significant survival advantage to tamoxifen (48 weeks versus 29 weeks) and a higher response rate in patients receiving DTIC and tamoxifen compared with DTIC alone (Cocconi & Bella et al., 1992). In another randomized clinical trial, Rusthoven et al of the National Cancer Institute of Canada (NCIC) conducted a double-blind, placebo-controlled trial comparing response rates and survival of 200 patients receiving the Dartmouth regimen with and without tamoxifen. There was no statistically significant difference between the two groups in either overall response rate or survival (Rusthoven & Quirt et al., 1996).

In a metaanalysis of published randomized controlled trials involved 912 patients, it has been demonstrated that tamoxifen does not improve the overall response rate, complete

response rate, or survival rate when administered along with combined chemotherapy or biochemotherapy regimens (Lens et al., 2003).

Single drugs used in metastatic melanoma have been summarized in table 1.

Drugs	Abbreviation	Number of patients	Dose	Overall response	References
Dacarbazine	DTIC	1868	250 mg/m ² /day x 5 d	15-25%	Hill 2nd et al., 1979
Temozolomide	TMZ	305	150-200 mg/m ² /day x 5 d	14%	Bleehen et al., 1995; Newlands et al., 1992
Carmustine	BCNU	122	75-110 mg/m ²	13-18%	Ahmann et al., 1976
Semustine	MET-CCNU	347	130 mg/m ²	16%	Ahmann et al., 1976
Fotemustine	FTMU	153	100 mg/m ² /week x 3 w	20-25%	Jacquillat et al., 1990
Cisplatin	CDDP	114	60-150 mg/m ²	15%	Glover et al., 1987
Carboplatin	CBDCA	30	400 mg/m ² iv every 4 weeks	19%	Evans et al., 1987
Vindesine	VDS	273	3 mg/m ² slow iv (7-14 day intervals)	14%	Quagliana et al., 1984
Vinblastine	VLB	62	6-8 mg/m ² slow iv 1/week	13%	Quagliana et al., 1984
Docetaxel	TXT	43	100 mg/m ² iv every 21 days	14%	Aamdal et al., 1994
Paclitaxel	TXL	34	125-275 mg/m ²	15%	Einzig AI et al., 1991
Tamoxifen	TAM	172	20 mg/day orally	7%	Rumke et al., 1992

Table 1. Single drugs used in metastatic melanoma

2.2 Combination chemotherapy

The role of combination chemotherapy in the treatment of metastatic melanoma remains uncertain. Historically there have been suggestions of improved activity with combination regimens, but reports of high response rates have generally emerged from single institution studies, and when large multicentre trials have been performed they have not confirmed these improvements (Chowdhury et al., 1999). There are a numerous combinations of chemotherapy for melanoma that have been are being developed and studied. These regimens have generally employed DTIC or, more recently, TMZ. Larger multiinstitution studies and results of randomized clinical trials strongly suggest that DTIC alone is as good as any of the combination regimens. Various combinations of DTIC, nitrosoureas, and cisplatin with other chemotherapeutic agents have been extensively evaluated in phase II

clinical trials, with response rates ranging from 20% to 40%. The more commonly tested combinations are presented in table 2.

A four-drug combination referred to as the BOLD regimen which includes bleomycin, vincristine, CCNU and DTIC was first studied regimen. Initial studies produced a response rate of 40%, with a 9% complete response rate. Follow-up phase II studies failed to confirm these results, with subsequent response rates falling to 4% to 20%. Another chemotherapeutic regimen extensively evaluated is the combination of vinblastine, cisplatin, and DTIC (CVD regimen), which was developed by Legha and colleagues. The response rates with this three-drug regimen range from 24% to 45% (Green & Schuchter, 1998). The Dartmouth regimen (CDBT) (McClay regimen) is a combination of cisplatin, carmustine, DTIC, and tamoxifen (NCCN Guidelines Version 2011 Melanoma, Treisman & Garlie, 2010).

Regimen	Doses	Response rate
BOLD	Bleomycin, 15 U day 1,4 Vincristine, 1mg/m ² day 1,4 CCNU, 80 mg/m ² day 1 DTIC, 200 mg/m ² day 1-5 28-day cycles	9%-40%
CVD	Cisplatin, 20 mg/m ² day 2-5 Vinblastine, 1.6 mg/m ² day 1-5 DTIC, 800 mg/m ² day 1 21-day cycles	24%-45%
CBDT (Dartmouth)	Cisplatin, 25 mg/m ² day 1-3 BCNU, 150 mg/m ² day 1 (given every other cycle) DTIC, 220 mg/m ² day 1-3 Tamoxifen, 20 mg/day 21-day cycles	19%-55%

Table 2. Combination chemotherapy regimens in metastatic melanoma

In a large phase III study comparing the CVD regimen to DTIC alone, there was a trend toward improved response and survival. The Dartmouth regimen originally resulted in a 55% response rate in the initial series of 20 patients with metastatic melanoma (Treisman & Garlie, 2010). A phase III multicentre trial that randomized 240 patients to the Dartmouth regimen vs dacarbazine monotherapy did not show a statistically significant benefit in favor of the combination. Despite a modest difference in objective response rate in favor of CDBT over DTIC (16.8% and 9.9%, respectively; $p=0.13$), there was no significant difference in overall survival (7.7 and 6.3 months, respectively; $p=0.52$). Myelosuppression, fatigue, nausea, and vomiting were significantly higher in the CDBT arm (Chapman et al., 1999).

Sileni et al. compared the activity and toxicity of the combination of dacarbazine, carmustine, cisplatin and tamoxifen (DBDT regimen) versus DTIC alone in patients with metastatic melanoma. Sixty patients were randomly assigned to receive BCNU 150 mg/m² intravenously on day 1, cisplatin 25 mg/m² iv. daily on days 1 to 3, DTIC 220 mg/m² iv

daily on days 1 to 3 and tamoxifen 160 mg orally daily for 7 days prior to chemotherapy (DBDT arm). Treatment cycles were repeated every 28 days, while BCNU was given every two cycles. The DTIC arm patients received DTIC alone 1200 mg/m² iv on day 1, repeated every 21 days. The overall response rate was 26% in the DBDT arm and 5% in the DTIC arm. Complete responses were 2.5% for DBDT and 0% for DTIC. The median progression-free survival and median survival were 4 and 9 months, respectively for DBDT, and 2 and 7 months for DTIC. DBDT was associated with significant haematological toxicity: 33% of the patients experienced a grade III or IV neutropenia and 28% a grade III or IV thrombocytopenia. The overall response rate obtained with DBDT was greater than that obtained with DTIC alone; however, this combination increased toxicity (Sileni et al., 2001).

The combination of paclitaxel and carboplatin (PC) has been reported to have antitumor activity in patients with metastatic melanoma, including patients who have received prior chemotherapy (Bhatia et al., 2009).

Zimpfer-Rechner et al. performed a randomized, multicentre, second-line clinical phase II study of paclitaxel either as monotherapy or combined with carboplatin given on an outpatient basis. In arm A, paclitaxel was administered at a dose of 100 mg/m² intravenously on day 1 each week for 6 weeks. In arm B, paclitaxel was administered at a dose of 80 mg/m² intravenously followed by carboplatin 200 mg/m² on day 1 each week for 6 weeks. The next cycle was administered after a 2 week intermission. The study was stopped after 40 patients because the overall response rate was below 10% in both arms. The median survival time after initiation of second-line treatment was 209 days for patients treated with paclitaxel only, and 218 days for those treated with paclitaxel/carboplatin. The median time to progression was around 56 days in both arms. Paclitaxel with or without carboplatin had only limited efficacy, and the combination of these drugs adds significantly to haematological toxicity without improving response or survival rates (Zimpfer-Rechner et al., 2003).

Rao et al. published their results with the combination of paclitaxel and carboplatin in 31 patients with metastatic melanoma. These patients had a median of two previous therapies, with the majority (29; 94%) having failed prior temozolomide or dacarbazine therapy. The most commonly used regimen was weekly paclitaxel (at a dose of 100 mg/m²) and carboplatin administered on days 1, 8, and 15 of a 28-day cycle. An objective partial response was noted in 8 patients (26%) with an additional 6 patients (19%) having stable disease; a clinical benefit was noted in 45% of those patients treated. The median time to disease progression was 3 months (range, 0-7 mos), with a median overall survival of 7.8 months (range, 1-14 mos). They concluded that the combination of paclitaxel and carboplatin appeared to have definite and clinically meaningful activity when used as second-line therapy after temozolomide or dacarbazine (Rao et al., 2006).

In a report on synthesis of randomized trials, 48 studies having 111 active treatment arms (24 with dacarbazine monotherapy, n=1390; 75 with dacarbazine combinations, n=4962; 12 with non-dacarbazine treatments, n=783) treating 7135 patients were examined. Response to dacarbazine monotherapy ranged between 5.3% and 28% (average 15.3%). Partial responses comprised 73% of successes. Only adding interferons improved response rates but survival duration was not significantly longer. All other treatments alone or in combination were ineffective (Lui et al., 2007).

A listing of major randomized studies evaluating DTIC versus drug combination is summarized in table 3.

Control arm dacarbazine dose/schedule	Study arm drugs (dose/schedule)	No. of randomised patients	Overall response (%)	Overall survival (months)	Study by
2 mg/kg/day (iv) × 10 days	Carmustine 150 mg/m ² (iv) + vincristine 2 mg/m ² (iv) on day 1 only	50	22 vs 25	NA	Bellet et al., 1976
250 mg/m ² (iv) on days 1- 5 every 3 weeks	Cisplatin 20 mg/m ² /day for 4 days starting on day 2 + vinblastine 1.6 mg/m ² /day × 5 days + dacarbazine 800 mg/m ² (iv) on day 1	104	11 vs 24	5 vs 6	Buzaid et al., 1993
1000 mg/m ² bid short iv infusion every 3 weeks	Tamoxifen 10 mg twice daily by mouth 1 week before chemotherapy +carmustine 150 mg/m ² on day 1+dacarbazine 220 mg/m ² (iv)+cisplatin 25 mg/m ² /days 1-3	240	10.2 vs 18.5	6.3 vs 7.7	Chapman et al., 1999
250 mg/m ² (iv) for 4 days every 3 weeks	Dacarbazine 250 mg/m ² (iv) days 1-4 every 3 weeks + detorubicin 120 mg/m ² iv every 3 weeks	51	15 vs 36	5 vs 6	Chauvergne et al., 1982
1200 mg/m ² day 1 every 3 weeks	Carmustine 150 mg/m ² (iv) on day 1 + cisplatin 25 mg/m ² (iv)/day on days 1-3 + dacarbazine 220 mg/m ² (iv)/day on days 1-3 + tamoxifen 160 mg orally/day × 7 days prior to chemotherapy. Treatment cycles repeated every 28 days, BCNU every 2 cycles	60	6 vs 26	7 vs 9	Chiarion Sileni et al., 2001
2.5 mg/m ² (I.V.) injection on days 1-4 every 4 weeks	Dacarbazine 2.5 mg/m ² (iv) by means of bolus injection on days 1-4 every 4 weeks + corynebacterium parvum 7 mg (im) 1 week before starting DTIC and at 4- week intervals thereafter	49	22 vs 27	5 vs 5	Clunie et al., 1980
250 mg/m ² on days 1-5 every 3 weeks	Dacarbazine 250 mg/m ² (iv) × 5 days, every 3- weeks + tamoxifen 20 mg/m ² orally daily	117	12 vs 28	11.8 vs 7.25	Cocconi et al., 1992

Control arm dacarbazine dose/schedule	Study arm drugs (dose/schedule)	No. of randomised patients	Overall response (%)	Overall survival (months)	Study by
200 mg/m ² (iv) for 5 days repeated every 3 weeks	po methyl-CCNU 200 mg/m ² once every 6 weeks Dacarbazine 150 mg/m ² (iv) × 5 days/3 weeks + po methyl-CCNU 130 mg/m ² 1/6 weeks	415	15 vs 15 15 vs 14	4.0 vs 4.2 4.0 vs 4.0	Costanza et al., 1977
250 mg/m ² (iv) on days 1- 5 every 3 weeks	Dacarbazine 250 mg/m ² (iv)/day on days 1-5 + epirubicin 90 mg/m ² on day 1 every 3 weeks	42	9 vs 21	NA	Lopez et al., 1984
250 mg/m ² (iv) on days 1- 10 every 4 weeks	Vinblastine 6 mg/m ² /day (iv) on days 1-2 + 24-h infusion of bleomycin 15 units/m ² from days 1-5 + cisplatin 50 mg/m ² 1 h (iv) infusion on day 5. After four courses, vinblastine and cisplatin were given alone. Courses repeated on a cycle of 4 weeks	77	14 vs 10	4.1 vs 3.42	Luikart et al., 1984
300 mg/m ² /day × 6 days every month days.	Dacarbazine 100 mg/m ² /8 h × 6 days every month days Carmustine 150 mg/m ² + vincristine 2 mg/m ² every 30 days	120	32 vs 29 32 vs 24	8.5 vs 8.4 8.5 vs 6.5	Moon et al., 1975
250 mg/m ² /day (iv) for 5 days every 4 weeks	Dacarbazine 250 mg/m ² /day (iv) × 5 days every 4 weeks + vindesine 3 mg/m ² /week	119	18 vs 25	4.1 vs 5.7	Ringborg et al., 1989
220 mg/m ² on days 1-3, q 21 days	Dacarbazine 220 mg/m ² on day 1-3 + carboplatine AUC 5, day 1, q 21 days	148	11.7 vs 21.3	7 vs 9	Babovic et al., 2008
200 mg/m ² /day (iv) for 5 days every 28 days	Arm 2: (I.V.) IFN-α 15 MU/m ² /day days 1-5 × 3 weeks, then (sc) 10 MU/m ² 3×/week + dacarbazine 200 mg/m ² daily (iv) days 1-5 starting on day 22, every 28 days Arm 3: orally tamoxifen 20 mg/day starting day 1 + dacarbazine 200 mg/m ² /day	280	15 vs 21 15 vs 18 15 vs 19	9.99 vs 9.33 9.99 vs 7.97 9.99 vs 9.54	Falkson et al., 1998

Control arm dacarbazine dose/schedule	Study arm drugs (dose/schedule)	No. of randomised patients	Overall response (%)	Overall survival (months)	Study by
	(iv) days 1-5 every 28 days Arm 4: (iv) IFN- α 15 MU/m ² /day days 1-5 \times 3 weeks, then (sc) 10 MU/m ² 3 \times /week + orally tamoxifen 20 mg/day starting day 1 + dacarbazine 200 mg/m ² /day (iv) days 1-5/28 days				
800 mg/m ² (iv) on days 1 and 21	Dacarbazine 800 mg/m ² (iv) days 1 and 21 + daily (im) INF- α 3 MIU at days 1-3, 6 MIU days 4-6, and 9 MIU daily thereafter. Started concomitantly Dacarbazine 800 mg/m ² (iv) days 1 and 21 + (IM) INF- α 3 MIU 3 \times /week. Started concomitantly	266	20 vs 28 20 vs 23	11 vs 13 11 vs 11	Bajetta et al., 1994
800 mg/m ² (iv) every 3 weeks	Dacarbazine (iv) escalating dose 200 mg/m ² , 400 mg/m ² , 800 mg/m ² /3 weeks; sc IFN- α starting at 3 MU/day on days 1-3, 9 MU/day on days 4-7, then 9 MU 3 \times /week	170	17 vs 21	7.36 vs 6.27	Thomson et al., 1993

Table 3. Key randomized studies evaluating dacarbazine (DTIC) vs drug combination

2.3 Biochemotherapy

Biochemotherapy, the combination of chemotherapy and biologic response modifiers, was developed in the early 1990s to improve response rates and durable remissions in metastatic melanoma. The initial regimens were given sequentially (chemotherapy followed by biologic response modifiers) because of concern of toxicity if all the drugs were given simultaneously. Paradoxically, sequential regimens were highly toxic because of the duration of treatment (10 to 14 days) and the combined and non-overlapping toxicities of chemotherapy and biologic response modifiers (O'day et al., 2002). An outpatient biochemotherapy regimen (carmustine, cisplatin, dacarbazine, tamoxifen, IL-2 and interferon with lower dose subcutaneous IL-2 and interferon) was developed by Thompson et al (Thompson et al., 1997). The first concurrent inpatient biochemotherapy regimen was developed by Legha et al. „Legha regimen“ combined cisplatin, vinblastine and dacarbazine (CVD) chemotherapy with continuous infusion IL-2 (9 MU/m² per day) for 4 days and 5

days of subcutaneous interferon alfa (5 MU/m² per day) at 21-day intervals. The results were encouraging with an overall response rate of 64%, a complete response rate of 21%, median survival of 12 months, and a 2-year survival rate of 10%. Efficacy was comparable to the inpatient sequential regimens, but the regimen was significantly less toxic. Fever/neutropenia occurred in 64% of patients and was the most significant reversible toxicity (Legha et al., 1998; O'day et al., 2002). The Legha regimen was subsequently modified by McDermott et al. to reduce toxicity. The modifications included reduction in the vinblastine dose, empiric granulocyte colony-stimulating factor (G-CSF) posttreatment, routine 5-HT₃ antagonist anti-emetic therapy, prophylactic antibiotics, frequent changes in central lines, dose reductions for toxicity, and limitation of treatment to a maximum of 4 cycles of therapy (McDermott et al., 2000). In a phase II trial with these modifications, toxicity was improved and the response rate was 48%, the complete remission rate was 20%, and the median survival was 11 months. Fever/neutropenia was not observed. G-CSF has now become a standard component of concurrent biochemotherapy regimens. Further modifications of the Legha regimen have been published with decrescendo dosing of continuous infusion IL-2. The rationale for decrescendo dosing of IL-2 is based on improved clinical response and reduced cumulative IL-2 toxicity (O'day et al., 2002).

Ridolfi et al. conducted a multicenter prospective randomized clinical trial in outpatients with metastatic melanoma to compare chemotherapy with biochemotherapy using immunomodulant doses of IL-2 and IFN α -2b. They randomized 176 patients with advanced melanoma to receive chemotherapy (cisplatin and dacarbazine with or without carmustine every 21 days) or biochemotherapy comprising the same chemotherapy regimen followed by low-dose subcutaneous IL-2 for 8 days and IFN α -2b three times a week, both for six cycles. At a median follow-up of 18 (chemotherapy) and 16 (biochemotherapy) months, median overall survival was 9.5 versus 11.0 months ($p=.51$), respectively. Treatment-related toxicity was fairly similar in both groups. They concluded that the addition of low-dose immunotherapy did not produce a significant advantage in overall survival, time to progression, or overall response (Ridolfi et al., 2002).

Bajetta et al. investigated the effects of additional cytokines to chemotherapy in 151 untreated metastatic melanoma patients. 75 patients received cisplatin 30 mg/m² on days 1-3, vindesine 2.5 mg/m² on day 1 and dacarbazine 250 mg/m² on days 1-3. 76 patients received same CVD scheme plus interferon- α 2b on days 1-5 and interleukin-2 on days 1-5 and 8-15, both administered subcutaneously, either recycled every 3 weeks. 10% of the patients were alive at a median of 52 months from start of therapy. They observed a response rate of 21% on arm A versus 33% on arm B; three patients (4%) given biochemotherapy had complete responses. Median time to progression was identical; median overall survival time was 12 months on arm A and 11 months on arm B. They also concluded that biochemotherapy was not better than chemotherapy alone, therefore biochemotherapy can not be recommended as standard first-line therapy for metastatic melanoma (Bajetta et al., 2006).

3. Novel targeted agents

The mitogen-activated protein kinase pathway plays a key role in melanoma development and is an important therapeutic target. Dysregulation of this pathway may result in increased signalling activity leading to proliferation, invasion, metastasis, migration,

survival and angiogenesis. Activating mutations in the BRAF and NRAS genes have been found to be relatively frequent in melanoma, occurring in approximately 50-60% and 15% of tumors, respectively (Lutzky, 2010). It has been shown that mutations in the KIT gene are more frequent in patients with melanomas arising from mucosal, acral and sun-damaged skin primary sites. NRAS and BRAF mutated melanomas are more commonly derived from non sun-damaged skin (Curtin et al., 2005; Curtin et al., 2006).

Recent reports describing major responses in KIT-mutated melanomas treated with imatinib mesylate and other drugs that inhibit KIT tyrosine kinase have led to larger trials of imatinib mesylate in mutation enriched populations, in an attempt to confirm that mutated KIT is a clinically important target in this small subpopulation of patients with melanoma. The most common BRAF mutation in melanoma (in 90% of BRAF-mutated melanomas) is the V600E mutation, which activates BRAF 500-fold. Sorafenib inhibits the BRAF serine/threonine kinase as well as various receptor tyrosine kinases, with significant activity in the VEGFR. Two randomized clinical trials testing sorafenib in combination with chemotherapy in melanoma produced negative results. The most likely explanation is that sorafenib is not very active against V600E mutated BRAF kinase. More specific BRAF-targeting drugs have been developed are under investigation. In a phase I trial recently published, PLX4032, an oral, selective inhibitor of oncogenic V600E BRAF kinase, induced complete or partial tumor regression in 81% of patients who had melanoma with the V600E BRAF mutation, with responses being observed in all sites of disease. Cutaneous side effects, fatigue and arthralgia were the most common side effects (Lutzky, 2010).

In a phase II study evaluating the effects of sorafenib in advanced melanoma, a total of 101 patients received placebo plus dacarbazine (n=50) or sorafenib plus dacarbazine (n=51). On day 1 of a 21-day cycle, patients received intravenous dacarbazine 1000 mg/m² for a maximum of 16 cycles. Oral sorafenib 400 mg or placebo was administered twice a day continuously. Median progression-free survival in the sorafenib plus dacarbazine arm was 21.1 weeks versus 11.7 weeks in the placebo plus dacarbazine arm (p=0.068). There were statistically significant improvements in progression-free survival rates at 6 and 9 months, and in time to progression in favour of the sorafenib plus dacarbazine arm. No difference in overall survival was observed. Sorafenib plus dacarbazine was well tolerated in patients with advanced melanoma and yielded an encouraging improvement in progression-free survival (McDermott et al., 2008). Hauschild et al reported a phase III randomized, placebo-controlled study on the efficacy and safety of sorafenib with carboplatin and paclitaxel in advanced melanoma who had progressed on a dacarbazine-or temozolomide-containing regimen. A total of 270 patients were randomly assigned to receive intravenous paclitaxel 225 mg/m² plus intravenous carboplatin at area under curve 6 (AUC 6) on day 1 of a 21-day cycle followed by either placebo (n=135) or oral sorafenib 400 mg (n=135) twice daily on days 2 to 19. The median progression-free time was 17.9 weeks for the placebo plus carboplatin arm and 17.4 weeks for the sorafenib plus carboplatin arm (p=.49). Response rate was 11% with placebo versus 12% with sorafenib. Grade III thrombocytopenia, diarrhea, and fatigue were more common in patients treated with sorafenib plus carboplatin versus placebo plus carboplatin. The addition of sorafenib to carboplatin did not improve any of the end points over placebo plus carboplatin in this study (Hauschild et al., 2009).

BAY 43-9006 is a novel RAF inhibitor that inhibits B-RAF and C-RAF. It is orally available and has been shown to be well tolerated. In a phase I/II trial of BAY 43-9006 in combination with carboplatin and paclitaxel, 35 melanoma patients were treated for at least 6 weeks. Among 32 evaluable patients, 11 (34%) had partial responses, including 10 ongoing at 3-16 months. Nineteen patients had stable disease as best response. The combination demonstrated activity in melanoma and had a favourable safety profile and no apparent pharmacokinetic interactions (Tarhini & Agarwala, 2006).

Angiogenesis and signalling through the ref/mitogen-activated protein/extracellular signal-regulated kinase/extracellular signal-regulated kinase cascade have been reported to play important roles in melanoma. Ref/mitogen-activated protein/extracellular signal-regulated kinase inhibitor AZD6244 is a new targeted agent for advanced melanoma (Friday & Adjei, 2008). Other important molecular pathways have been found to be altered in melanoma, opening new avenues for therapeutic intervention. These include the phosphatidylinositol-3-kinase, microphthalmia-associated transcription factor, cyclin-dependent kinases, notch-1 and iNOS pathways. Early clinical trials with drugs that are active in these pathways are being conducted (Lutzky, 2010).

4. Conclusion

Metastatic melanoma has remained refractory to systemic treatment for decades. Single-agent or combination chemotherapy or biologic response modifiers alone have not resulted in response rates of durable remissions that are high enough to affect median survival. In the past decade, biochemotherapy regimens have been developed that appear to produce systemic response in approximately 50% patients and durable remissions in 10% to 20%. Modified concurrent biochemotherapy regimens have preserved efficacy and reduced toxicity, thus allowing for larger community-based clinical trials that are currently ongoing. These trials will determine the role of biochemotherapy as first-line treatment for metastatic disease. Further understanding of the molecular and immunologic mechanisms that promote survival of melanoma tumor cells will undoubtedly lead to the development of better, more specific and perhaps less toxic agents.

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Chemocentric Chemoimmunotherapy: A New Concept in Melanoma Immunotherapy

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1. Introduction

According to the American Cancer Society, 68,130 new cases of melanoma and 8,700 deaths were predicted for 2010 (Jemal et al., 2010). Although melanoma comprises only 5% of all skin cancers, it is by far the deadliest, responsible for 75% of skin cancer-related deaths, and its incidence is steadily increasing worldwide. Primary cutaneous melanoma has an excellent outcome with early surgical intervention, but regional and distant metastatic disease have a much more dismal prognosis. Current five-year survival rates equal 91% for all disease sites, including 98% for local disease, 62% for regional disease (including regional lymph node involvement), and only 15% for those with distant metastasis (median survival is only 7.5 months). Unfortunately, for those patients who will develop locally advanced and metastatic melanoma, current treatment options are limited in scope and effectiveness; thus, the overall poor mortality rates for these patients emphasize the need for better treatment modalities, methods of relapse prevention and earlier recognition of pre-neoplastic and neoplastic cells (Atallah & Flaherty, 2006; Balch et al., 2001a, 2001b; Blesa et al., 2011).

Currently, there are no curative therapies for metastatic melanoma. Until March 25, 2011, the U.S. Food and Drug Administration (FDA) had only approved two drugs for the treatment of metastatic melanoma, the DNA alkylating agent dacarbazine and high-dose Interleukin-2 (IL-2). Dacarbazine alkylates and crosslinks DNA in all phases of the cell cycle, impairing DNA function, inducing cell-cycle arrest and apoptosis (Bajetta et al., 2002), and is a common chemotherapeutic option for multiple cancers (Seam et al., 2009; Yang & Chapman, 2009). IL-2 activates endogenous anti-tumor-reactive T cells and natural killer (NK) cells. Notably, circulating NK cells from stage IV melanoma patients were recently shown to exhibit a unique phenotypic and functional character toward melanoma cells, and this unique character was altered in patients after treatment with chemotherapy (Fregni et al., 2011). Dacarbazine induces objective tumor regression in 15-20% of metastatic melanoma patients with a median survival of only 8-9 months and a 2-3% complete response rate, and single agent bolus IL-2, while providing a 20-30% objective response rate, creates significant long-term response in only 5% of patients treated, is fraught with toxicity and costly. Combination low-dose IL-2 and chemotherapy and single-agent high-dose IL-2 produce a

similarly low complete response (Atkins et al., 2000; Tsao et al., 2004). Other cytotoxic compounds have resulted in poor response rates and significant adverse effects, including temozolomide, cisplatin, carboplatin, vinca alkaloids, taxanes, and nitrosoureas (Bafaloukos et al., 2002; Bajetta et al., 2002; Blesa et al., 2011; Khayat et al., 2002; Middleton et al., 2000). Notable agents of promise currently undergoing testing are anti-CD40, anti-PD-1L, 1-MT (1-methyl-D-tryptophan), and the BRAF-inhibitor PLX4032 (Bollag et al., 2010; Comin-Anduix; Flaherty et al., 2010; Fonsatti et al., 2010). On March 25, 2011, the U. S. Food and Drug Administration approved ipilimumab (Yervoy™, Bristol-Myers Squibb Company, New York, NY, USA), a monoclonal antibody against the inhibitory lymphocyte receptor, CTLA4, as an injection for the treatment of unresectable or metastatic melanoma (Hodi et al., 2010).

Advanced melanomas are infamously resistant to chemotherapy, principally due to the presence of inherent primary anti-apoptotic and acquired cellular chemoresistance mechanisms (Serrone & Hersey, 1999). Of note, a recent study by Rosner et al. (Rosner et al., 2011) described a means to circumvent such apoptosis resistance. Without activating the apoptosis signaling cascade or utilizing other apoptotic nucleases, recombinant deoxyribonuclease I (DNaseI), engineered with a nuclear localization signal and mutated actin-binding site to avoid entering its inactive bound form, was effective in inducing apoptosis in an apoptosis-resistant melanoma cell model with 70-100% killing efficiency, emphasizing its potential application in many of the known apoptosis-resistant cancers. Surgical resection of primary and known metastatic lesions is still the most effective means of improving overall survival of melanoma patients, though palliative care is standard for patients with metastases after first-line dacarbazine or other physician-preferred therapy, as survival is very rare (Rass et al., 2008; Tagawa et al., 2006; Yang et al., 2006). Surgery in partnership with radiation is standard for locoregional control, while additional chemical-, cell- or antibody- based therapies, or some combination thereof, are commonly attempted for systemic eradication of tumor cells in the blood or at unspecified sites in the body to combat current disease and prevent relapse and metastatic implantation. Melanoma has attracted a lot of interest from immunologists due to a heightened frequency over other solid tumors to display fast, spontaneous, complete tumor regression associated with a specific cellular immune response (Kadison & Morton, 2003). Chemoimmunotherapy, tumor vaccines and other chemotherapeutic combinations have shown improvement in objective response rates for melanoma patients, but they have not yet been able to show significant survival benefits (Bajetta et al., 2006; Eton et al., 2002; Kaufmann et al., 2005). Indeed, a recent meta-analysis of 18 studies comprising 2,625 participants revealed improved objective response rates in metastatic melanoma patients treated with chemoimmunotherapy compared with chemotherapy, but no improvement was noted in overall survival benefit (Sasse et al., 2007). Additionally, hematological and non-hematological toxicities were greatest in those undergoing chemoimmunotherapy.

While the historic chemoimmunotherapeutic approach has been to sensitize tumor cells to immunotherapy with administration of chemotherapeutic compounds, we have described a high degree of chemosensitization with pre-treatment of non-tumor-Ag-specific CD4+ T cells (Radfar et al., 2009), our termed 'chemocentric chemoimmunotherapy' strategy. This technique may provide a basis for development of novel methods of selectively reducing the chemoresistance of virtually all tumor cell types, given the lack of requirement for tumor-Ag-specific T cells. We will present an overview of conventional chemoimmunotherapy development for melanoma and examine the functional significance of chemocentric chemoimmunotherapy for melanoma and other cancers.

2. Background

It has long been part of the clinical canon that chemotherapy drugs negatively affect immunological systems, inhibiting anti-tumor immune activity by a variety of related mechanisms. However, the intricacies of the chemo-immuno interaction provide areas of opportunity for utilizing chemo drugs to enhance the immune response. These cytotoxic drugs tend to target dividing lymphocytes, necessary for development of an immune response, but they also deplete CD4+/CD25+ regulatory T cells (T reg), potentially enhancing the response. Again, conversely, lymphodepletion triggers homeostatic T cell reconstitution which proceeds to create T cells recognizing tumor as self. Opportunities arise during the depletion of T reg and the heightened formation of T cells, allowing an avenue to both infuse the patient with tumor-reactive T cells which are now not impeded by T reg and the potential to create within the patient a large quantity of T cells reactive to some tumor antigen(s).

Conventional cancer chemotherapy is capable of inducing the death of immunologically sensitive tumor cells by utilizing the host innate and adaptive immune responses, e.g. through the induction of immunoregulatory cytokines (Bracci et al., 2007), and some of these drugs can alter cellular pathways of immune suppression and tolerance in a dose- and time-dependent manner, e.g. inducing homeostatic proliferation (Bracci et al., 2007; Proietti et al., 1998), or modulate the extracellular release of certain proteins upon cell death, e.g. HMGB1 release and subsequent tumor survival/metastasis-promoting inflammation is inhibited by oxaliplatin (Dong et al., 2007). Additionally, some chemo drugs can modulate the expression of tumor antigens and antigen processing/presentation machinery on tumor cells. For example, 5-Fluorouracil (5-FU) has been shown to induce carcinoembryonic antigen (CEA) expression in colon and breast cancer cells (Correale et al., 2003) while 5-aza-2'-deoxycytidine can induce the expression of cancer testis antigens and the cell surface MHC class I complex in melanoma and other cancers (Adair & Hogan, 2009; Coral et al., 2002; Fonsatti et al., 2007; Natsume et al., 2008). Notably, direct intralesional injection of the MHC class I complex, which allows tumor immune evasion when defective in numerous cancers (Lampen & van Hall, 2011; Maleno et al., 2011), via high-dose Allovectin-7® (Vical Inc., San Diego, CA, USA), a cationic lipid-formulated bicistronic plasmid encoding MHC-I components β -2 microglobulin and HLA-B7, in 127 recurrent or previously unresponsive to chemotherapy stage III and IV melanoma patients tested for efficacy in a recent phase II dose escalation study produced an objective response of 11.8% with median duration of response of 13.8 months (Bedikian et al., 2010). Tissue from two responding patients was noted for the absence of melanoma upon histological analysis, and the therapy was well tolerated.

Drugs of complementary activity and dissimilar toxicities are routinely combined to enhance individual anti-tumor effects. In the 1960s, effective combination antibiotic therapy for tuberculosis influenced the combination treatment strategy of acute lymphocytic leukemia and lymphoma. A number of significant combination chemotherapeutic regimens were developed in the 1980s, including CVD (cisplatin, vinblastine, and dacarbazine) and the Dartmouth regimen (cisplatin, carmustine, dacarbazine, and tamoxifen) (Del Prete et al., 1984; Legha et al., 1989). Notably, according to two independent clinical trials of the 1990s, CVD and Dartmouth were not shown to significantly increase overall survival relative to dacarbazine alone (Buzaid et al., 1993; Chapman et al., 1999). Although dacarbazine and cisplatin-based combination chemotherapy/biochemotherapy regimens combining IFN

and/or IL-2 are now recommended by the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology for Melanoma for stage III and IV melanomas, promising increases in response rates notwithstanding (Riker & Howell, 2010a, 2010b), significant improvement in long-term responses has yet to be seen.

Early efforts to combine chemotherapy with immunomodulators, particularly cancer vaccines, were discounted given the understood detrimental myelosuppressive properties of many chemotherapeutic compounds (Emens, 2008). This suppression was purported to be short-lived and reversible, as the replenishing of leukocytes following chemotherapy would show, by Harris et al. in 1976 (Harris et al., 1976). As reviewed by Sinkovics and Horvath (Sinkovics & Horvath, 2006), around this time, the M.D. Anderson Hospital Melanoma-Sarcoma Service submitted multiple grant applications to the NCI for combination chemoimmunotherapeutic intervention strategies for patients with metastatic melanomas and sarcomas. Despite NCI rejection, clinical trials began, utilizing the findings of Lindenmann and Klein (Lindenmann & Klein, 1967) from nearly a decade earlier which showed an acquired immunity to tumor formation in mice injected with the cell culture lysates of oncolytic myxo- or paramyxovirus-infected tumor cells. Sinkovics et al. further adapted this strategy for the post-chemotherapy administration of cultured tumor cell lysates infected with oncolytic, attenuated PR8 influenza A virus. PR8 viral oncosylates, the immunotherapeutic suspension, were comprised of UV-irradiated, endotoxin-free tumor cell cultures with low levels of live PR8 virus. 'Tumor-specific active immunization' with viral oncosylates two-to-three weeks post-chemotherapy resulted in heightened rates of remission with moderate, occasionally significant increases in survival.

Early phase clinical trials have been able to show response rates of up to 50% for chemoimmunotherapies (Atkins et al., 2003; Eton et al., 2002; Keilholz et al., 1997; Rosenberg et al., 1999); however, the duration of responses has been limited in most cases to less than a year. Importantly, systemic toxicities and adverse side effects tend to be heightened with chemoimmunotherapeutic interventions in relation to chemotherapy or immunotherapy alone. Furthermore, many of the most common chemotherapies were not inherently designed to take into account the entire tumor-host microenvironment and the effects of such therapies in conjunction with locally and systemically active immunological mechanisms. Designing treatment course strategy according to the character of the tumor-host microenvironment milieu is vital for its long-term efficacy (Formenti, 2010; Pages et al., 2009; Zitvogel et al., 2008). Today, thirty years after the first modern chemoimmunotherapy clinical trials were initiated, the impasse that is melanoma chemoresistance has continued to stymie significant, clinically-applicable advancement in this therapeutic field. Given the recurring roadblocks of current treatment strategies, traditional methods of chemotherapy and immunotherapy use are being radically adjusted to achieve a better overall clinical outcome.

3. Conventional chemoimmunotherapy for the treatment of melanoma

Immunotherapy can play an important role in the treatment of cancer, and combinations of chemotherapy and immunotherapy have been investigated in many clinical trials. Although some phase III trials have shown mixed results regarding duration of survival, meta-analyses have found no improvement in overall survival (Allen et al., 1998; Ives et al., 2007; Keilholz et al., 1998; Sasse et al., 2007). Chemoimmunotherapy, or biochemotherapy, has been extensively studied in melanoma. Conventional chemoimmunotherapeutic strategies

include two major categories. The first category uses cytokines such as IL-2 and/or IFN- α in combination with chemotherapy. This experimental strategy was not supported by strong preclinical evidence and has not been shown to be superior to chemotherapy alone (Atkins et al., 2003; Eton et al., 2002; Keilholz et al., 1997, 2005; Rosenberg et al., 1999). The second category is a strategy that we have termed 'immunocentric chemoimmunotherapy'. In this approach, the primary or central focus is on immunotherapy, with chemotherapy playing a peripheral role in immunomodulation. Using this approach, melanoma patients have been successfully treated with non-myeloablative, lymphodepleting chemotherapy and adoptive cell transfer with tumor-Ag specific lymphocytes and high dose IL-2 (Dudley et al., 2002, 2005). Although this strategy is promising, it is not widely applicable to non-melanoma cancers due to the difficulty in generating large numbers of tumor-Ag specific tumor-infiltrating lymphocytes. To date, there have been no studies using chemoimmunotherapy that have shown a significant improvement in overall survival (Atkins et al., 2003, 2008; Bajetta et al., 2006; Eton et al., 2002; Kielholz et al., 1997, 1998, 2005; Middleton et al., 2007; Punt et al., 2006; Ridolofi et al., 2002; Rosenberg et al., 1999), and the development of immunotherapy for the treatment of a broad range of cancer types is still lacking. Given the proven capacity of many already developed and thoroughly tested, well characterized chemotherapeutic agents to have significant, specific and intricate influence over global and localized immune response, adjustments to the multitudes of chemoimmunotherapeutic strategic fronts are currently being made in anticipation of a clinically relevant breakthrough.

3.1 Cytokines and chemotherapy

Activated T cells amplify cytokines that are essential for an effective immune response (Szabo et al., 2003). These include IL-2, TNF- α , IL-21 (by activated CD4+ T cells), GM-CSF, IFN- γ , and other as yet unidentified cytokines. In addition to their immunomodulatory activities, some cytokines can also have direct effects on tumor cells and/or tumor vasculature. TNF- α is known to induce hemorrhagic necrosis in tumors (Carswell et al., 1975; Old, 1985) and has also been shown to induce apoptotic and necrotic tumor cell death *in vitro* (Helson et al., 1975; Laster et al., 1988; Rubin et al., 1988; Sugarman et al., 1985). The hemorrhagic necrotic effect of TNF- α has been shown to be more potent when used in combination with chemotherapy in a rat sarcoma model (Manusama et al., 1996). However, in these studies, TNF- α had no direct activity against tumor cell lines *in vitro* and demonstrated no synergy with chemotherapy, acting instead on the tumor vasculature (van Horssen et al., 2006; Watanabe et al., 1988). The IFNs can exert direct effects on the proliferation, differentiation, and apoptosis of tumor cells (Chawla-Sarkar et al., 2001, 2003; Detjen et al., 2001; Gooch et al., 2000; Pfeffer et al., 1998). However, the response to the IFNs varies considerably, depending on the tumor histology, and resistance to the IFNs has been reported in several tumor types (Burke et al., 1999; Harvat & Jetten, 1996; Kaplan et al., 1998; Phan-Bich et al., 1997).

Several early-phase trials of cytokines combined with cisplatin-based therapies have shown an overall response rate of ~50%, though such responses are of very short duration and often complicated by a high degree of systemic toxicity and adverse side effects. Over the past 15 years, numerous cisplatin-based cytokine combination regimens have been assessed (Atkins et al., 2002, 2003; Atzpodien et al., 2002; Buzaid et al., 1993; Flaherty et al., 2001; Legha et al., 1989, 1998; McDermott et al., 2000; Pejcic et al., 2010; Rosenberg et al., 1999,

2011; Thompson et al., 1997). Notably, in a phase II trial from 1997, Thompson et al. describe the development and utility of an outpatient chemoimmunotherapy treatment regimen of monthly cycles of intravenous cisplatin, carmustine, dacarbazine, and tamoxifen plus self-administered subcutaneous recombinant IL-2 and IFN- α for 32 metastatic melanoma patients, 30 assessed for clinical response (Thompson et al., 1997). After three months, they note a complete response rate of 13% and a 30% partial response, and toxicity was primarily restricted to minor fever and nausea with only a few instances of hospitalization for toxicity management.

Rosenberg et al. (Rosenberg et al., 1999) reported in 1999 on their phase II clinical trial testing tamoxifen, cisplatin, and dacarbazine in 52 metastatic melanoma patients, while the 50 patients in the other arm received the same therapy followed by IL-2 and IFN- α 2b. The chemoimmunotherapy arm exhibited 44% total response rate while the chemotherapy arm was 27%; however, of the seven complete responders, only three received the cytokines, indicating no significant impact of cytokines on complete response, and median duration of response was less than six months. Even more disappointing, most of the toxicities were reported for the chemoimmunotherapy group. Median duration of survival was 10.7 months for those receiving cytokines compared to 15.8 months for those without. A regimen of concurrent treatment with CVD chemotherapy, IL-2 and low-dose IFN- α has shown to be better tolerated and more practical than sequential treatment of CVD followed by IL-2/IFN- α (Atkins et al., 2002; Eton et al., 2002; Legha et al., 1998), though randomized phase III trials of this CVD/IL-2/IFN- α combination show no improvement in response or survival over CVD and modified CVD chemotherapy regimens alone (Atkins et al., 2003; Keilholz et al., 2005). In regard to pre-treatment of a chemoimmunotherapeutic regimen with chemotherapy, Punt et al. (Punt et al., 2006) reported on a randomized, phase II clinical trial of metastatic melanoma patients receiving either dacarbazine, cisplatin, IFN- α and IL-2 or the same combination after an initial treatment of two cycles of dacarbazine, finding no difference between the two groups in terms of median overall survival.

O'Day et al. (O'Day et al., 2009) treated 133 chemotherapy-naïve metastatic melanoma patients with CVD, decrescendo IL-2, and IFN- α 2b with granulocyte-macrophage colony-stimulating factor (GM-CSF) cytokine support. Maintenance biotherapy with low-dose IL-2 and GM-CSF followed by intermittent pulses of decrescendo IL-2 over 12 months was provided for those without disease progression. The overall response rate was 44% with 8% complete response and 36% partial response. Stable disease was noted for 29% of patients; median progression-free survival was 9 months, and median survival was 13.5 months with 12-month and 24-month survival rates at 57% and 23%, respectively, providing evidence of above average long-term survival rates for these patients. Further randomized trials need to be carried out.

A recent multicenter phase II chemoimmunotherapy trial of the Dermatologic Cooperative Oncology Group (DeCOG) testing the multi-kinase inhibitor sorafenib in combination with pegylated-IFN- α 2b (PEG-IFN- α 2b) in 55 patients with metastatic melanoma resulted in two patients with partial response and 14 with stable disease (29.1% disease control rate in intent-to-treat population) (Egberts et al., 2011). Median progression-free survival was 2.5 months, and toxicities were primarily hematological, including one treatment-related bleeding complication leading to death. A smaller study of 22 metastatic melanoma patients receiving a regimen of CVD with PEG-IFN- α 2b, subcutaneous IL-2 and oral 13-cis-retinoic acid, with maintenance biotherapy for those with clinical response after six courses,

provided 12 objective responses with seven maintenance-receiving patients having stable disease after six months (Recchia et al., 2008). Median progression-free and overall survivals were 23.3 and 45.7 months, respectively, with mostly hematological toxicities and 21% of patients exhibiting grade 2 autoimmune reactions.

3.2 Immunocentric chemoimmunotherapy

Another conventional strategy is one that we have termed 'immunocentric chemoimmunotherapy'. This strategy is an immunotherapy-focused approach which uses chemotherapy as an immunomodulator to enhance the effect of cancer vaccines or adoptive cell transfer therapy. Based on this approach, recent studies using chemotherapy to prepare the host before the infusion of *ex vivo* activated, melanoma Ag-specific tumor-infiltrating lymphocytes and high dose IL-2 have resulted in impressive response rates.

3.2.1 Cancer vaccines

Cancer vaccines are promising for their abilities to elicit both humoral (antibody) and cellular (T cell) immune responses (Finn, 2008; Schoenfeld et al., 2010) specifically against tumor cells, as well as for their minimal side effects and the potential for development of immunologic memory allowing for a long-term, durable response to treatment without the need for continuous therapy (Yannelli & Wroblewski, 2004). The presence of cell surface antigens and receptors, capable of activating a myriad of intracellular biochemical pathways, makes possible the development of cancer vaccines with highly stringent criteria for target tumor cell selection and may assist in the focused delivery and activity of chemotherapeutic agents. The recent FDA-approval of sipuleucel-T (Provenge®; Dendreon Inc., Seattle, WA, USA), the first personalized therapeutic cancer vaccine, for the treatment of advanced prostate cancer, has opened the doors for novel vaccine development for other cancers, contributing to the heightened interest in targeted immune-based study of cancer systems.

Nistico et al. (Nistico et al., 2009) recently reported on their phase I/II pilot study testing 36 HLA-A2+ disease-free, stage II to IV melanoma patients with standard-dose dacarbazine administered one day before vaccination with HLA-A2 restricted melanoma antigen A (melan-A/MART-1) and gp100 melanoma peptide vaccination compared with vaccination alone. Dacarbazine significantly increased the numbers of peptide-specific CD8+ effector memory T cells which recognize and lyse HLA-A2+/melan-A+ tumor cells, indicating the enhancement of effective CD8+ T cell recognition of vaccine peptides after treatment with a chemotherapy drug in patients with melanoma. Another more recent pilot clinical trial (Palermo et al., 2010) by the Nistico group examined the role of dacarbazine again as pre-treatment to this vaccine therapy, noting a progressive widening of the T cell receptor repertoire diversity with concomitant high avidity and tumor reactivity in melan-A-specific T cell clones of patients treated with this chemoimmunotherapy and a trend toward longer survival. Alternatively, patients receiving vaccine alone exhibited a tendency to narrowing the T cell receptor repertoire diversity and a decrease of tumor lytic activity in one patient.

3.2.2 Adoptive cell transfer

One of the most exciting avenues for melanoma therapy is adoptive cell transfer of tumor-reactive T cells harvested from the tumor itself, expanded and activated *ex vivo* using various methods (e.g., high concentrations of IL-2) and then transferred back into the

patient. The success of adoptive cell transfer depends on the specificity of T cells for the tumor and their ability to survive and proliferate in the environment. Notably, intratumoral injection of toll-like receptor agonists were found to enhance the potency of activated adoptive cell transfer against a murine model with established subcutaneous melanoma tumors, acting to enhance IFN- γ production and subsequent killing of the now more immunogenic tumor cells by adoptively transferred T cells (Amos et al., 2011). Metastatic melanoma patients have benefited in certain settings with adoptive cell transfer of tumor-infiltrating lymphocytes in combination with chemotherapeutic lymphodepletion, with an impressive objective response rate of 50-70% (Dudley & Rosenberg, 2007; Gattinoni et al., 2006; Hershkovitz et al., 2010; Khattar et al., 2009; Rosenberg et al., 2008, 2011; Rosenberg & Dudley, 2009). Given the promising results of adoptive cell transfer strategies in melanoma and other cancers, many notable contributions are being made toward a more discrete understanding of this field.

During expansion of tumor-infiltrating lymphocytes for infusion, CD8⁺ T cells are driven to differentiate into effector cells, losing key costimulatory molecules such as CD28 and CD27. Costimulation with CD137/4-1BB was recently shown to significantly increase lymphocyte survival during melanoma adoptive cell transfer and improve the anti-tumor response (Hernandez-Chacon et al., 2011). Inozume et al. (Inozume et al., 2010) reported on the recovery of function of PD-1 cell surface receptor protein on CD8⁺ T cells in melanoma digests after *ex vivo* conditioning with IL-2, leading to the production of much more tumor-specific IFN- γ compared with PD-1⁻ T cells and the proposal of PD-1 functional operation or presence being a capable biomarker for selecting tumor-specific lymphocytes from melanomas. As a common practice in adoptive cell transfer prior to infusion of these lymphocytes, patient lymphodepletion of T reg and other lymphocytes in competition for cytokines allows for a much more potent response to activated tumor-specific T cells (Dudley et al., 2002, Wrzesninski et al., 2010). Adoptive cell transfer with autologous tumor-infiltrating lymphocytes, conditioned with cyclophosphamide and fludarabine, and IL-2 tested in 50 metastatic melanoma patients selected to receive either 2-Gy (non-myeloablative) or 12-Gy (myeloablative) of total body irradiation resulted in response rates of 49%, 52% and 72% for patients not receiving radiation and those receiving either 2- or 12-Gy, respectively (Dudley et al., 2008). The authors note the possibility for increased tumor-infiltrating lymphocyte activity, proliferation, and/or persistence in the body and at the tumor site as a result of rising cytokine availability following irradiation, having seen a significant increase of IL-7 and IL-15 in the serum following the myeloablative regimen. This trial is part of three whose combined and complete results were recently published by Rosenberg et al. (Rosenberg et al. 2011), providing impressive objective response and overall survival rates. In a panel of 93 heavily pretreated metastatic melanoma patients (86% with visceral metastases and 95% recurring after prior therapy), they found adoptive cell transfer of autologous lymphocytes plus high-dose IL-2, administered within one day following a lymphodepletion regimen of cyclophosphamide and fludarabine or either 2- or 12-Gy of total body irradiation, to produce 52 objective responses (56%) with 20 (22%) complete responses, 19 of which lasted a span of three to seven years.

Other clinical studies have utilized genetically engineered peripheral T cells in place of tumor-infiltrating lymphocytes. Due to limitations in generating tumor-specific T cells for adoptive cell transfer, Morgan et al. (Morgan et al., 2006) reported on the specific conference of tumor recognition by autologous lymphocytes from peripheral blood of 15 metastatic melanoma patients using a retrovirus that encodes a T cell receptor recognizing a tumor

antigen. Adoptive cell transfer of these transduced cells after lymphodepletion resulted in durable engraftment of more than 10% of peripheral blood lymphocyte levels for over two months after infusion. In two patients demonstrating objective regression of metastatic melanoma lesions, circulating engineered cells were maintained at high levels at one year after infusion.

Such viral T cell receptor engineering is currently being studied for advanced melanoma and general (Chinnasamy et al., 2010) cancer therapy by Dr. Steven A. Rosenberg's group and colleagues in The National Cancer Institute's Surgery Branch (Rosenberg & Dudley, 2009). Indeed, they have recently described the selective delivery of IL-12, noted for its limited immunostimulatory clinical application due to toxicity, into the tumor microenvironment upon tumor-Ag recognition by T cell receptors on genetically engineered lymphocytes, enhancing *in vivo* melanoma tumor regression without toxicity (Zhang et al., 2011). Another recent report from this group describes adoptive cell transfer of autologous T cells with a T cell receptor against the NY-ESO-1 cancer/testis antigen, expressed in ~25% of patients with melanoma and other epithelial cancers, resulting in an objective response in five of 11 patients with NY-ESO-1-expressing tumors, with two complete responses of greater than one year (Robbins et al., 2011).

3.3 Cyclophosphamide for melanoma immunocentric chemoimmunotherapy

Cyclophosphamide has been an invaluable chemotherapeutic agent in studying immunocentric methodology and application. Numerous reports have shown the synergistic capabilities of cyclophosphamide in combination with immunotherapeutics, describing its ability to induce cytokine expression, type I IFNs and homeostatic proliferation of B and T cells and to deplete the T reg population from the tumor site, increasing the possibility of CD8+ T cell-mediated targeting and destruction of tumor cells (Berd et al., 1986, 1990, 2004; Bracci et al., 2007; Dummer et al., 2002; Ercolini et al., 2005; Ghiringhelli et al., 2004; Livingston et al., 1987, 1994; Maine & Mule, 2002; North, 1982; Nowak et al., 2006; Proietti et al., 1998; Schiavoni et al., 2000; Turk & Parker, 1982; Zitvogel et al., 2008). An important study in 2005 showed non-myeloablative lymphodepleting chemotherapy with cyclophosphamide and fludarabine conditioning of tumor-infiltrating lymphocytes followed by infusion and subsequent high-dose IL-2 treatment to produce a 51% overall response rate in 35 metastatic melanoma patients previously having received IL-2 therapy, all but one being refractory to IL-2 (Dudley et al., 2005).

A surge of recent studies have expounded on the role of cyclophosphamide in T reg modulation and immune therapies that can take advantage of the T reg depleted tumor environment. Combination cyclophosphamide and an agonist antibody against the co-stimulatory CD4+ Foxp3+ T reg cell receptor OX40 has recently been shown to induce apoptosis of the tumor T reg population and induce the influx of CD8+ T cells resulting in a potent anti-tumor response in an *in vivo* B16 melanoma model (Hirschhorn-Cymerman et al., 2009). Furthermore, Kohlmeyer et al. (Kohlmeyer et al., 2009) reported chemotherapeutic preconditioning with cyclophosphamide prior to adoptive cell transfer and viral vaccination followed by adjuvant peritumoral injections of immunostimulatory nucleic acids to be a highly effective chemoimmunotherapeutic regimen, resulting in complete regression of primary and lung metastatic lesions in the normally adoptive cell transfer-resistant genetically engineered Hgf-Cdk4R24C metastatic melanoma mouse model.

In following the combination of chemotherapy and adoptive cell transfer with immune-enhancing cytokines, such as IL-2 (Dudley et al., 2002, 2005; Dudley & Rosenberg, 2007;

Mihalyo et al., 2004), and the recent characterization of IL-21 with adoptive cell transfer by Hinrichs et al. (Hinrichs et al., 2008), another study describes the boosting of immunocentric therapeutic efficacy with IL-21 (Petersen et al., 2010), an immune-enhancing cytokine that, unlike IL-2, does not support proliferation of activated T reg nor activation-induced cell death (Spolski & Leonard, 2008; Waldmann, 2006). As collective data would indicate, IL-21 likely enhances the T reg-depleting capabilities of cyclophosphamide by abrogating T reg development, via the inhibition of IL-2 secretion, and function (Elsaesser et al., 2009; Fantini et al., 2007; Hinrichs et al., 2008; Peluso et al., 2007; Piao et al., 2008; Yi et al., 2009). Mice with established tumors, subsequently treated with cyclophosphamide and adoptive cell transfer and/or daily injections of IL-21, exhibited better early tumor growth inhibition up to five days after the last (day 7) IL-21 injection and increased circulating tumor-specific T cells with the IL-21 administration. The authors note the potential for continued tumor growth stunting with continued IL-21 injection as well as the importance of timing considerations for this particular design (Skak et al., 2009). A similar study by Salem et al. (Salem et al., 2007) showed a more pronounced effect using peptide vaccination and naïve T cells, possibly a more effective option than pre-primed cells for adoptive cell transfer (Hinrichs et al., 2008).

4. Chemocentric chemoimmunotherapy

We have developed a third strategy for combining chemo- and immunotherapy, termed 'chemocentric chemoimmunotherapy' (Radfar et al., 2009). In this model, chemotherapy plays the central effector role, while immunotherapy is used to sensitize the tumor and its microenvironment to the cytotoxic effect of chemotherapy. This strategy employs the use of nonspecifically activated CD4+ T lymphocytes (aCD4) as a chemosensitizer of tumor cells followed by treatment with chemotherapeutic drugs. The rationale for this strategy is based on the known ability of activated T cells to secrete multiple cytokines that can regulate proliferation and/or apoptosis of tumor cells and the ability of activated T cells to exert direct activity on tumor cells through apoptotic pathways such as the Fas/Fas ligand pathway; for instance, tumor cell apoptosis is initiated when the Fas ligand, present on activated T cells, binds the Fas receptor, present on tumor cells. This model does not depend on Ag-specific activation of T cells and is, therefore, potentially applicable to a wide variety of tumor types and patients regardless of their HLA status. Indeed, preclinical studies examining the role of chemocentric chemoimmunotherapy in melanoma, breast, colon and prostate cancer cell lines have shown a dramatic enhancement of induced tumor-cell apoptosis. With the added advantage of ease of use and cost effectiveness in the clinic, chemocentric chemoimmunotherapy is a promising future methodology that is widely applicable, in terms of patient character schema, cost and basic technical utility, in the clinical setting.

4.1 Chemosensitization with nonspecifically activated CD4+ T cells

According to conventional wisdom, it is counterintuitive to administer chemotherapy immediately after cell therapy because the transferred immune cells will be eliminated by chemotherapy. Although this may be true in the setting of conventional immunocentric chemoimmunotherapy, where immunotherapy plays the major effector role and chemotherapy is used to prepare the host, this approach is rational in the context of chemocentric chemoimmunotherapy, where chemotherapy exerts the principal effector function and immunotherapy is used transiently for the purpose of presensitization.

We have recently demonstrated that presensitization of tumor cells with nonspecifically activated CD4⁺ T cells (aCD4) greatly enhanced the cytotoxic effect of chemotherapy in both *in vitro* and *in vivo* models (Radfar et al., 2009). This activity was observed for all seven tumor cell lines as well as all four chemotherapeutic agents tested. Soluble factors secreted from the activated CD4⁺ T cells were found to be responsible for the observed effect, with IFN- γ playing a major role in the chemosensitization of tumor cells. IFN- γ by itself, however, was consistently inferior to activated CD4⁺ T cells in the chemosensitization of tumor cells.

For three human metastatic melanoma, two human breast, one prostate and one colon cancer cell line, treated with 5-FU, temozolomide (TMZ), carboplatin (Carbo), or paclitaxel, chemosensitization with aCD4 had a dramatic impact on tumor cell viability and *in vivo* tumor formation. TMZ, the oral imidazotetrazine derivative of dacarbazine that is also capable of crossing the blood-brain barrier, ultimately produces the same dacarbazine compound after intake and has been shown in clinical trial to be similarly effective in treating metastatic melanoma as dacarbazine (Middleton et al., 2000). Treatment of the A375 melanoma cell line with TMZ after presensitization with aCD4 resulted in near complete cell death, with residual cell viability as low as 5%, compared to 67% viability for cells treated with TMZ that were not presensitized with aCD4. Similar study of two more melanoma cell lines provided comparable results. Further tests using Carbo, a drug with minimal activity against melanoma, resulted in a decrease of melanoma cell viability of ~83% at the optimal aCD4 concentration, compared with no change in viability after Carbo treatment alone. Non-melanoma cell lines treated with paclitaxel, Carbo or 5-FU all gave similar results.

Presensitization was found to be mediated in large part via cytokines, principally IFN- γ , and not through cell-cell contact. aCD4 supernatants were screened for 13 common Th1 and Th2 pro- and anti-inflammatory cytokines. IL-1b, IL-5, and IL-12p70 levels were negative or minimally expressed. The remaining 10 were tested for in combination against aCD4 alone for their chemosensitizing ability, assessed based on viability after TMZ treatment. There was no increase in chemosensitizing ability of combination IL-2/-4 over cells without cytokine addition, and combinations IL-6/-8 and IL-10/-12p40/-13 produced enhanced activity of 7% and 8%, respectively. Combination IFN- γ /TNF- α /GM-CSF significantly enhanced the cytotoxic activity of TMZ by 17%. While most individual cytokines provided little to no enhancement of TMZ cytotoxicity, IFN- γ in combination with other cytokines consistently provided the greatest impact on chemosensitization, and IFN- γ combinations showed no improvement over IFN- γ itself; however, the effects of IFN- γ by itself or in combination lagged the single combination of all 10 tested cytokines. Administration of aCD4 alone prior to TMZ treatment as a positive control yielded the best results, showing significant enhancement of TMZ cytotoxic cell killing over the 10-cytokine combination.

Lysates from A375 cells presensitized with aCD4 followed by treatment with TMZ were analyzed via a transwell system for cytoplasmic histone-associated DNA fragments as a measure of apoptosis. Melanoma cells treated with TMZ alone or aCD4 alone resulted in slightly higher levels of DNA fragments, ~49 arbitrary units (AU) and 54 AU, respectively, as compared 23 AU for nontreated cells. However, when A375 cells were presensitized with aCD4 and treated with TMZ, the level of DNA fragments detected, 1041 AU, was more than 21-fold over that of the TMZ alone group, 19-fold over the aCD4 alone group, and 45-fold over the nontreatment control. In addition, a significantly higher level of caspase-8 activity, ~2300 RFU, was detected in cells in the experimental group compared with the control

groups, i.e., no treatment (524.4 ± 3.5 RFU), TMZ treatment only (508.6 ± 28.3 RFU), or aCD4 treatment only (392.7 ± 4.0 RFU). A reduction in the relative expression of Bcl-2 protein was also detected in A375 cells treated with aCD4 alone or the combination of aCD4 followed by TMZ, but not in cells treated with TMZ alone. These data demonstrated that presensitization of tumor cells with aCD4 led to a decrease in Bcl-2, and that treatment with aCD4 and TMZ together resulted in enhanced caspase-8 activity, resulting in significant reduction in cell viability through an increase in apoptosis. The combination of aCD4 and chemotherapy yielded dramatic results at 24 hours in viability experiments. To evaluate true pro-apoptotic potential of aCD4 presensitization, apoptosis assays were performed at earlier time points, 12 hours instead of 24 hours after treatment with chemotherapy. For caspase-8 activity, chemotherapy treatment was stopped after 5 to 6 hours. Even at these early time points when aCD4 alone or chemotherapy alone had no discernable effects, the combination of aCD4 and chemotherapy resulted in a dramatic increase in apoptosis and caspase-8 activity. Two human tumor xenograft models provided evidence of significant tumor growth delay for melanoma and breast cancer after presensitization with aCD4 and subsequent treatment with TMZ or paclitaxel, respectively. The melanoma xenograft also displayed significant growth inhibition. In the first model, injections of aCD4 were made intratumorally, followed 48 hours later with intraperitoneal administration of TMZ, to athymic nude mice bearing human melanoma A375 xenograft. There was a significantly pronounced delay in tumor growth compared with mice receiving no treatment or either treatment alone. Similarly, mice bearing aggressive MDA-MB-231 human breast tumors treated with aCD4 followed by paclitaxel had significant delay in tumor growth compared with each of the control groups. The difference between aCD4 plus paclitaxel versus paclitaxel alone was statistically significant. Interestingly, aCD4 alone had a significant growth inhibition effect on the melanoma xenograft but not on the breast cancer xenograft. This was consistent with the known immuno sensitivity of melanoma compared with other solid tumors. To assess toxicity resulting from the experimental treatment, animal weights were measured and recorded during the course of the study. No statistically significant difference in weight was found between the experimental group and control groups up to day 30 after initiation of treatment.

Some recent studies may help shed light onto the possible mechanisms that underlie the dramatic chemosensitizing effect of aCD4. Inflammation and inflammatory cytokines, as represented by a mixture of IL-1 β , TNF- α , and IFN- γ , were shown to generate nitric oxide through the induction of nitric oxide synthase, resulting in global inhibition of DNA repair activity in cholangiocarcinoma cells (Jaiswal et al., 2000). Perrotta et al. (Perrotta et al., 2007) further described chemosensitization through dendritic-cell-mediated intratumoral administration of nitric oxide in a B16 mouse melanoma model. Other studies demonstrated that IFN- β or IL-24 could overcome TMZ resistance in neuroblastoma and melanoma, respectively, through down-regulation of the DNA repair enzyme O6-methylguanine-DNA methyltransferase expression and activity (Rosati et al., 2008; Zheng et al., 2008). Additionally, several recent clinical studies have reported improved response rates and survival with salvage chemotherapy in patients who previously received cancer vaccination and developed an immune response (Antonia et al., 2006; Arlen et al., 2006; Gribben et al., 2005; Schlom et al., 2007; Wheeler et al., 2004). Our model may provide a plausible mechanism to explain these observations as well as to facilitate further understanding, design, and development of improved methods for chemoimmunotherapy in cancer.

5. Conclusion

Despite intense research efforts, scientists are still struggling with the simplest principles of tumor cell proliferation, cell cycle kinetics and the genetic basis of malignant transformation and tumor progression. In particular, treatment options for melanoma are few, and those with metastatic disease have been afforded only meager therapeutic options in the past, maintaining a disappointing 15% five-year survival rate. In somewhat of a contradiction, melanoma is now at the forefront of targeted and immunological therapeutic study, evident by the surging number of relevant publications and the recent addition of ipilimumab to a short list of FDA-approved therapies for metastatic melanoma patients. One of the most popular and theoretically promising of these directions currently being studied is combination chemoimmunotherapy.

While many chemoimmunotherapeutic regimens have resulted in lackluster responses, our novel adaptation of this methodology for melanoma and other cancers, utilizing non-tumor-specific activation of CD4+ T cells as a chemosensitizer, has provided exciting results. We have shown dramatic increases in the efficacies of four functionally unique chemotherapeutic drugs in seven cell lines comprising four different cancer types with our 'chemocentric chemoimmunotherapeutic' strategy. After presensitization with aCD4, cytotoxicity was greatly enhanced for all chemo drugs, both *in vitro* and *in vivo*, against all cell types tested. In addition, we recently described the presensitization of tumor cells with aCD4 prior to gamma-irradiation to significantly enhance cancer cell growth inhibition (Wang et al., 2010). Soluble factors released by aCD4, particularly IFN- γ , were primarily responsible for the observed activity, and TNF- α , though inactive by itself, significantly augmented the radiosensitizing activity of IFN- γ .

Not only are the time and monetary costs of producing non-tumor-specific activated CD4+ T cells far and away less than the current standard of activating against a specific set of patient tumor cell antigens, but, as our data shows, aCD4 may also be applied in patients with a variety of cancer types in synergistic conjunction with the most currently effective and applicable chemotherapy for the individual patient. Regardless of the cell line used or its sensitivity or resistance to a particular chemotherapeutic drug, in all our tested cases, presensitization with aCD4 greatly enhanced the cytotoxicity of the drugs, resulting in near complete cell death. Therefore, patients with a typically chemoresistant cancer, such as metastatic melanoma, may be able to benefit from enhanced chemotherapeutic efficacy with aCD4 presensitization. Additionally, utilization of a much broader range of chemotherapeutic agents may be possible for patients who might normally have only a small number of available options, specific for their tumor type and physical condition. Patients may thus be able to receive treatment sooner, accessing the readily available wealth of well-studied chemotherapeutic drugs without the need to await new drug development and testing. To this end, drug selection may ultimately be better tailored to patient tolerance, creating a therapeutic strategy with greater anti-tumor activity, therefore, less time for development of chemoresistance, and better tolerance which will allow more patients to remain on their particular regimen for its appropriate duration. Indeed, the utilization of this novel technique may be able to provide a more effective and efficient means to combat melanoma and other cancers.

6. References

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Melanoma Immunomodulation: A War of Attrition

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1. Introduction

Melanoma is an aggressive skin cancer that has an occurrence rate of about 1 in every 50 Americans (Giblin & Thomas, 2007). It is the 6th most common cancer, with a lifetime risk of 2.04% for men and 1.38% for women (Jemal et al., 2008; Jilaveanu et al., 2009). According to the American Cancer Society, in 2009 the number of new cases of melanoma rose to 68,720 with 8,650 new deaths attributed to the disease (ACS, 2010). There are many known factors that contribute to the formation of melanoma. One major factor influencing the increase in melanoma cases is increased exposure to ultra-violet radiation (UVR). Other risk factors include skin type, hair/eye color, the presence of dysplastic nevi and/or increased nevi, and a family history of melanoma (Jilaveanu et al., 2009). Also, mutations in BRAF, CDKN2A, and CDK4 genes have all been attributed to melanoma development. BRAF mutations have been found in 70% of all melanomas and greater than 90% of these mutations carry a single missense (single nucleotide change) mutation (Meyle & Guldborg, 2009). CDKN2A is involved in melanoma pathogenesis and is a germline mutation found in younger patients (Liu et al., 2007). CDK4 is involved in cell-cycle arrest and has been identified in 10% of melanomas (Bennett, 2008). These mutations are mostly detected in non-chronic sun-induced damage melanomas, while chronic sun-induced damage melanomas are more commonly observed.

When melanoma arises, early diagnosis is crucial to survival. With early diagnosis, more than 80% of cases can be treated successfully through surgery (Zhu et al., 2009). This surgery includes excision of the tumor and surrounding tissue; lymph nodes near the tumor may also be removed if evidence of metastasis is present. Other treatments for melanoma include radiation and chemotherapy, used particularly in cases of highly aggressive and metastatic disease (ACS, 2010). Side-effects from these treatments include fatigue, malaise, and an increased susceptibility to non-melanoma cancers (Kamposioras et al., 2010). Also, these therapies are severely toxic to the patients, suggesting a need for improved, less toxic treatment options that specifically target melanoma tumors, like immunotherapy. The multiple ways that melanoma alters the immune system locally, at the site of the tumor, and systemically makes the disease difficult to treat but ideal for the study of immunomodulation (Berinstein, 2009). Immunomodulation, the alteration of the immune system or its function, is exploited in multiple forms by melanoma tumors from changes in the cellular and sub-cellular makeup of the tumor to changes in the tumor microenvironment that suppress localized and systemic attempts at disease reduction.

At the cellular level, melanoma tumors differentially express cytokines, chemokines, and soluble molecules responsible for immunosuppression and tumor proliferation which will be discussed further in this chapter, particularly those with potential for targeting or with therapeutic benefits (Lazar-Molnar et al., 2000). Melanoma cells are also less efficient in antigen (Ag) presentation to CD4+ T cells, reducing immune detection of melanoma tumors and the effectiveness of some immunotherapy strategies (Norton & Haque, 2009). Multiple defects along the HLA class II pathway are present in melanoma cells, the alteration of which could prove useful in novel tumor targeting and immunotherapeutic vaccination strategies. These defects and the potential to overcome them will be further explained in this chapter. Costimulatory molecules are also altered in melanoma cells, reducing positive cellular interaction with T cells and professional antigen presenting cells (APCs), while promoting immunosuppressive interactions through CD28, CTLA-4, and the B7 family of immune inhibitors (Pardee et al., 2009; Wolchok & Saenger, 2008). Study focused on enhancing these secondary stimulation signals would promote complete T cell stimulation and activation of anti-tumor CD8+ T cells, a current goal of most immunotherapy strategies. Melanoma cells are also capable of modulating the surrounding immune cells including: suppression of tumor infiltrating lymphocytes (TILs), enhancement of CD4+CD25+FoxP3+ T regulatory cells (Tregs), increased immature myeloid suppressor cells, increased pro-tumorigenic m2 macrophages, and generation of melanoma-associated fibroblasts (Oble et al., 2009; Camisaschi et al., 2010; Balsamo et al., 2009; Ilkovitch & Lopez, 2008). These topics will be further dissected throughout the chapter as they relate to both immune suppression and multimodal treatment strategies. The course of tumor progression not only adds more problems to immune regulation of the disease, but also more potential targets for therapeutic intervention. Melanoma angiogenesis and metastasis aggravate immune suppression in distinct and specific ways increasing the morbidity and mortality of the disease, while reducing the effectiveness of current treatment options (Schadendorf et al., 2009; Zbytek et al., 2008; Mahabeleshwar & Byzova, 2007). Both of these topics will be further examined with specific emphasis on the relationship between Interleukin (IL)-8, vascular endothelial growth factor (VEGF), the matrix metalloproteinases (MMPs), and the distinct problems faced when tumors metastasize, particularly to lymph nodes, the lungs, liver, and brain (Sloan et al., 2009; Vahrmeijer et al., 2008; Huang et al., 2008; Yang et al., 2009).

Melanoma also represents one of the most widely studied tumors in terms of immunotherapy design, clinical evaluation, and therapeutic application (Jandus et al., 2009). This chapter will discuss the successes and failures of melanoma immunotherapy strategies like IL-2 therapy, melanoma vaccines like canvaxin, and adoptive cell transfer in terms of immunomodulation and its effect on treatment (Atkins, 2006; Goldman & DeFrancesco, 2009). This chapter will also discuss current strategies being developed and potential new directions in treatment that address the immunomodulatory nature of melanoma. These include combined chemoimmunotherapy, melanoma monoclonal antibodies, and multimodal therapy strategies (Kudo-Saito et al., 2005; Ascierto et al., 2010; Flaherty, 2006). The goal of this chapter is to summarize the multiple roadblocks in melanoma treatment associated with the immunomodulation instigated by melanoma tumors. By understanding these issues, novel targets for melanoma therapy can be developed and the shortcomings of current treatment modalities can be enhanced leading to improved patient care and patient outcomes. The fight against melanoma in many ways is a war of attrition: gradual gains can and must be made by making treatment more effective through improved knowledge of the immunomodulatory mechanisms that melanoma tumors employ.

2. The tumor microenvironment

When discussing the ability of melanoma tumors to induce both local and systemic immunomodulation, it is important to first understand the tumor microenvironment itself, and how the alterations at this level affect both tumor progression and limit the effectiveness of some treatment strategies. Melanoma arises through a complex process of cellular mutation and a loss of keratinocyte control over melanocyte growth and differentiation (Hsu et al., 2002; Shirakata, 2010). This imbalance leads to the formation of early stage nevi, appearing localized near the basement membrane of the skin. As malignant melanoma progresses, it develops through interaction between dysfunctional melanocytes and the tumor microenvironment. The progression from nevocellular nevi to dysplastic nevi is accompanied with changes in both keratinocytes and local adhesion molecules allowing for increased melanocyte-melanocyte interaction and the formation of nevocyte nests at the dermal-epidermal junction (Danen et al., 1996; Hsu et al., 2002). Following this development, melanocytes fail to respond to keratinocyte or epidermal cell signaling, they no longer form dendrites, and start to modulate the immune environment through the release of cytokines and immune activation factors which will be described in this chapter (Ilkovitch & Lopez, 2008). Tumors next proceed through two distinct growth phases, radial and vertical growth, accompanied by increased inflammation, immune modulation, and healthy cell destruction. In this sense, the abuse of the immune system drives the progression of disease to a more aggressive phenotype, again through shed factors which will be further explained in this chapter. Finally, following vertical growth through the basement membrane of the dermis following periods of high angiogenesis, melanoma cells are now free to metastasize to local and distant sites resulting in poor disease prognosis (Ria et al., 2010). This section will also focus on the distinct ways melanoma cells respond to the tumor microenvironment during the course of melanoma progression, and how these alterations could be exploited in developing novel melanoma therapies.

2.1 Shed molecules and immunosuppression

At the cellular level, melanoma tumors differentially express cytokines, chemokines, and soluble molecules responsible for immunosuppression and tumor proliferation. Initially, these molecules can have regulatory roles in the tumor microenvironment through growth inhibition, but these functions are lost as tumors slowly progress to a state of localized immune suppression (Lu & Kerbel, 1993). Table 1 is a brief summary of some of the cytokines and growth factors associated with melanoma progression and immunosuppression. A more thorough examination of these factors is expertly presented in a review by Ilkovitch et al (Ilkovitch & Lopez, 2008).

Some cytokines appear to have dual roles within the tumor microenvironment depending on the stage and advancement of disease. During initial tumor formation the inflammatory cytokine IL-6 shed by localized keratinocytes, epithelial, and immune cells inhibits tumor proliferation. IL-6-induced growth inhibitor during early stages of melanoma garnered some attention as an immunological target, but clinical application failed to show any benefit (Lu & Kerbel, 1993). During late stages of disease, IL-6's control of over-growth is lost and autocrine usage of IL-6 produced by melanoma cells actively enhances tumor progression through the STAT3 pathway, which can be further enhanced through interactions with IL-17 (Wang et al., 2004; Hodge et al., 2005; Wang et al., 2009). Elevated STAT3 activity regulates tumor oncogenic factors, cell survival, and cell proliferation

Molecule	Role
IL-1(α and β)	Melanoma derived IL-1 α and IL-1 β induce fibroblast and endothelial growth factors as well as surface adhesion molecules allowing for the growth and metastasis of melanoma cells, also can stimulate IL-6 production.
IL-6	Initial tumor suppression, then stimulates tumor growth through autocrine regulation by activating Stat3, IL-17 can also be induced in this system further stimulating Stat3.
IL-8	Highly involved in angiogenesis through chemoattraction of infiltrating lymphocytes and cell adhesion regulation.
IL-10	Anti-inflammatory, induces T cell and DC suppression, can be excreted by tumors and by tolerized or regulatory T cells.
IDO	Inhibits T lymphocyte mediated antigen-specific immune responses through suppression of tryptophan, also promotes immune tolerance.
FasL	Melanoma cells lack functional FasL, which prevent FasL interaction with Fas receptor on lymphocytes, and modulate apoptosis induction.
TGF- β	Multiple roles in immunosuppression, can be secreted by melanoma tumors acting in both autocrine and paracrine manner, converts immune cells to suppressive regulatory phenotype.
PGE2	Released from melanoma associated fibroblasts, inhibits NK T cell activity and adds to immunosuppression.

Table 1. Immunomodulatory Molecules Influencing Melanoma Growth

molecules resulting in angiogenesis, tumor growth, and in some cases (i.e. brain) metastasis (Xie et al., 2006). Similarly to IL-6, TGF- β also displays growth inhibitor paracrine function during early stages of disease, and autocrine tumor growth in later stages of progression (Ma et al., 2009; Pardali & Moustakas, 2007).

Anti-inflammatory cytokines and immunomodulatory molecules are often exploited by melanoma cells, most notably IL-10. Melanoma cells, melanoma recruited myeloid suppressor cells, and Tregs actively secrete IL-10 to induce tolerized T cells and dendritic cells (DC) (Huang et al., 1999; Polak et al., 2007). The chemokine IL-8 also plays a major role in melanoma progression, particularly in angiogenesis. Autocrine produced IL-8 can stimulate melanoma growth and induce expression of cellular adhesion molecules allowing for tumor cell migration. The chemoattractant nature of IL-8 also allows for the recruitment of monocytes and macrophages to the tumor site which release growth factors modulating vascular permeability contributing to cell migration. The tumorigenic properties of IL-8 are well summarized by Waugh and Williams (Waugh & Wilson, 2008). Based on these characteristics, targeting IL-8 could reduce the angiogenic nature of melanoma cells allowing for improved clearance of tumors, an area that should be further studied for its therapeutic potential. Additionally, two shed molecules indoleamine-2,3-deoxyginase (IDO) and prostaglandin E2 (PGE2) also contribute to melanoma induced immunosuppression and represent interesting potential targets in immunotherapy design. IDO is produced primarily by suppressive lymphocytes and immature myeloid/dendritic cells. IDO also acts as a tryptophan sink in the tumor microenvironment, severely inhibiting T cell activation (Polak et al., 2007; Honig et al., 2004). Recent study suggests that PGE2 is produced by melanoma-associated fibroblasts and immature myeloid cells and aids in the recruitment of a specific lineage of migratory DC with low cytokine expression profiles (Luft et al., 2002). PGE2 also inhibits NK T cell anti-tumor activity contributing to immunosuppression in the tumor microenvironment (Balsamo et al., 2009). Taken together, these molecules represent hurdles to

most immunotherapy strategies employed, which rely on the state of the immune system at the time of treatment to overcome the tumor burden. Steps should be taken to inhibit PGE2 and IDO, as well as the suppressive cytokines mentioned prior to immunotherapy, allowing for a more robust immune response. These cytokines are not solely responsible for the deficiency in current treatment strategies; the cellular makeup of TILs and the melanoma-associated neighboring cells may play major roles in immune subversion.

2.2 Immune cells

As seen in the description of shed molecules, there is dynamic crosstalk between tumors and surrounding tissues and infiltrating immune cells that result in both tumor challenge and in tumor progression. Often, autocrine and paracrine signaling pathways between melanoma cells and surrounding cells contribute to tumor progression and metastasis as reviewed by Lazar-Molnar et al (Lazar-Molnar et al., 2000). The shift from tumor suppression to tumor progression and metastasis results, in part, from the alteration in the type and characteristics of TILs. These changes include the enhancement of CD4⁺CD25⁺FoxP3⁺ Tregs, increased immature myeloid suppressor cells, increased pro-tumorigenic m2 macrophages, and the generation of melanoma-associated fibroblasts (Kalluri & Zeisberg, 2006; Mantovani et al., 2002; Almand et al., 2001; Bronte et al., 2001). From a treatment perspective, understanding the alterations in immune cells localized to the tumor site provides both the reason for the failures of some immunotherapy and some novel ways to treat the disease. As stated previously, the progression from healthy melanocyte to melanoma occurs through both mutations within the tumor and through alterations of the cellular environment around the melanoma. In the skin, tissue homeostasis is critical in cellular regulation as well as immune control, and melanoma tumors disrupt this regulation through multiple processes.

Melanoma cells release high levels of basic fibroblast growth factor (bFGF) which results in the generation and localization of melanoma-associated fibroblasts to the tumor microenvironment (Meier et al., 2000). These cells are unlike normal skin fibroblasts in that they proliferate rapidly and eventually outnumber most other cell types within the tumor microenvironment (Li et al., 2003; Lee et al., 2005). They also release molecules that support the growth and movement of melanoma tumors in the extracellular matrix (ECM) of surrounding tissue and affect the function of NK T cells, aiding in immune inhibition (Balsamo et al., 2009). Melanoma tumors also recruit immune suppressor cells such as immature dendritic cells, myeloid derived suppressor/immature myeloid cells, and M2 macrophages (Kusmartsev & Gabrilovich, 2006; Bronte et al., 2001; Hanson et al., 2009). These cells work together releasing immunosuppressive molecules like TGF- β , IL-1 α , IDO, IL-10, PGE2, and reactive oxygen species (ROS) (Kusmartsev & Gabrilovich, 2003; Almand et al., 2001; Valenti et al., 2006). Functionally, these cells are deficient in melanoma tumor antigen presentation, resulting in indirect tumor tolerance through interaction with CD8⁺ T cells (Almand et al., 2001). These factors work against the anti-tumor infiltrating lymphocytes creating a network of immune suppression surrounding the tumor, and through localized inflammation and dysregulation of cell adhesion molecules, they aid in tumor growth, movement, and angiogenesis (Brigati et al., 2002). Thus, targeting these cells in combination with melanoma tumors should improve the efficacy of immunotherapy strategies, such as targeting shared signaling pathways between tumors and surrounding tissue like STAT3 and BRAF/MAPK (Sumimoto et al., 2006; Inamdar et al., 2010).

Figure 1 illustrates the cells melanoma tumors induce for their benefit and the effect that they have on anti-tumor immunity. When discussing melanoma immunosuppression, one cannot leave out the role that T cells play in the tumor microenvironment, in particular the detrimental role of FoxP3+ Tregs, reviewed by Oble et al (Oble et al., 2009). Tregs are normally involved in regulating immune responses to avoid autoimmunity and in reigning in the cytolytic effects of effector CD8+ T cells. They represent the suppressive arm of CD4+ T cells, and are best identified by their high expression of CD25 and FoxP3 (Camisaschi et al., 2010; Vence et al., 2007). In melanoma, particularly in advanced disease states, Tregs are the primary infiltrating lymphocyte where they directly inhibit any cytotoxic antitumor activity through direct contact inhibition, and the release of high levels of IL-10 (Baumgartner et al., 2007). Study suggests that high serum concentrations of Tregs are associated with poor prognosis, poor treatment responses, and an increased risk of recurrence (Vence et al., 2007).

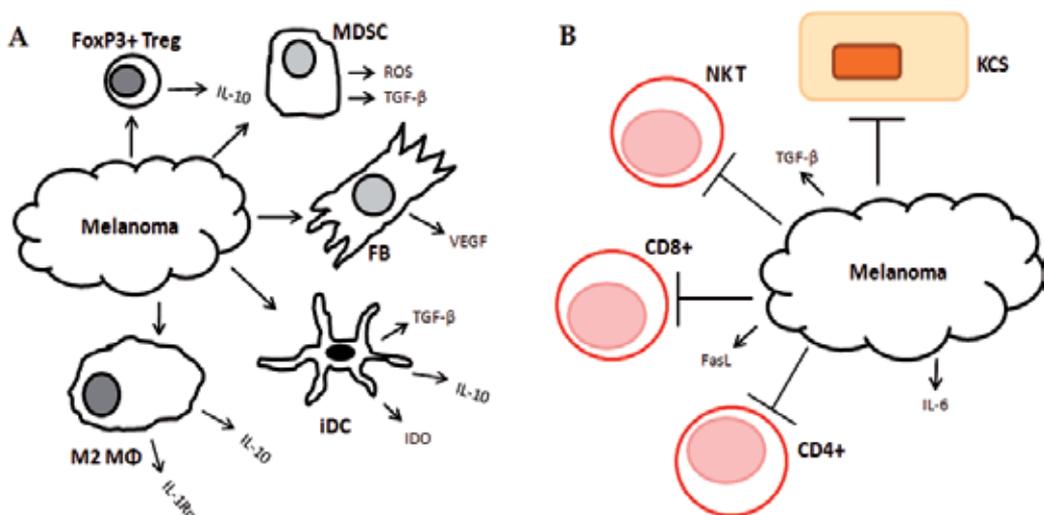


Fig. 1. Microenvironment and immune cell dysfunction. A. Melanoma cells recruit melanoma-associated epithelial and immune cells. These include fibroblasts (FB), immature dendritic cells (iDC), myeloid derived suppressor cells (MDSC), M2 macrophages (M2 MΦ). And CD4+CD25highFoxP3+ T cells (FoxP3+Treg) which release molecules like IL-10, IL-13R α , IDO, TGF- β , VEGF, and ROS that can inhibit antitumor activity and promote tumor growth. B. Melanoma cells directly interfere with limiting endothelial and immune cells like keratinocytes (KCS), NK T cells (NK T), cytotoxic lymphocytes (CD8+), and CD4+ effector cells (CD4+) through the expression of TGF- β , shed and surface FasL, and IL-6.

Tregs are also found in high numbers in sentinel lymph nodes and peripheral blood in cases of metastatic melanoma where they interfere with the expansion of CD8+ and CD4+ effector cells through IL-2 suppression (Viguier et al., 2004). Study in mice revealed that Treg depletion resulted in the expansion of highly reactive CD8+ T cells resulting in tumor clearance, and in human studies, depleting lymphocytes prior to adoptive cell transfer (ACT) improved the effectiveness of treatment (Mahnke et al., 2007; Matsushita et al., 2008). These results could be associated with the deletion of the large pool of Tregs in the tumor microenvironment and represent an important immunotherapeutic option going forward.

The multiple ways of targeting these immunosuppressive cells in therapy design will be highlighted later in this chapter.

3. Melanoma antigen processing and presentation

In conjunction with these observed deficiencies in the tumor microenvironment leading to immune dysfunction, it is important to understand the direct interplay between melanoma tumors and the immune cells attempting to regulate and destroy tumors. Much focus has been paid to the suppressive nature of Tregs, yet melanoma cells have their own mechanisms of directly inhibiting CD8⁺ cytotoxic and CD4⁺ effector T cells (Viguier et al., 2004; Lampen & van Hall, 2011; Norton & Haque, 2009). CD8⁺ and CD4⁺ T cells interact with melanoma tumors through contact with HLA class I and HLA class II molecules on their cell surface, respectively. Multiple defects exist in melanoma cells ranging from complete loss of class I and II expression to subversive Ag generation attributed to defects in endosomal/lysosomal machinery. These issues and how they represent novel mechanisms for disease treatment and immunotherapy design will be discussed in this section.

3.1 Antigen processing and presentation

The general consensus when describing immunological strategies against melanoma is in the induction of a cytotoxic immune response mainly generated by the activation of anti-tumor CD8⁺ T cells. Though CD8⁺ T cells perform the bulk of the tumor destruction, by focusing solely on activating these cells and not CD4⁺ T cells, melanoma tumors are capable of devising strategies to avoid CD8⁺ T cell detection and activation. Clinical evidence supports this notion, as even in patients with advanced disease there are detectable CD8⁺ T cells specific for melanoma tumor Ag, yet the tumor remains unchallenged (Harlin et al., 2006). This occurs through multiple mechanisms from the immunosuppression described in the previous sections of this chapter, and also through flaws in melanoma Ag processing, presentation, and costimulation. Melanoma tumors have also been shown to downregulate HLA class I surface expression, preventing any T cell activation and tumor clearance (Lopez-Nevot et al., 1988; Cabrera et al., 2007). Tumors also differentially express costimulatory molecules required for complete T cell activation, which will be discussed further in the next section of this chapter. An additional reason for the inability of CD8⁺ T cells to clear a tumor completely is that there are few to no support signals driving the anti-tumor immune response further following the initial activation and a complete lack of potent antitumor immunological memory. The support signals needed are supposed to come from activated CD4⁺ effector T cells, differentiated from Tregs by their low CD25 expression and the lack of FoxP3 (Lizee et al., 2006). CD4⁺ T cells release immune stimulatory cytokines and can directly cross present Ag to professional antigen presenting cells (APCs), driving a complete immune response that can lead to the development of immunological memory. CD4⁺ T are also crucial as they interact with HLA class II on melanoma tumors which present self tumor Ags (Figure 2). However, melanoma cells are severely hindered in their ability to present endogenous tumor Ags (Goldstein et al., 2008). Although melanoma cells can reduce their HLA class II, studies have shown that detectable levels of surface class II are still present that could be exploited in immunotherapeutic vaccine design.

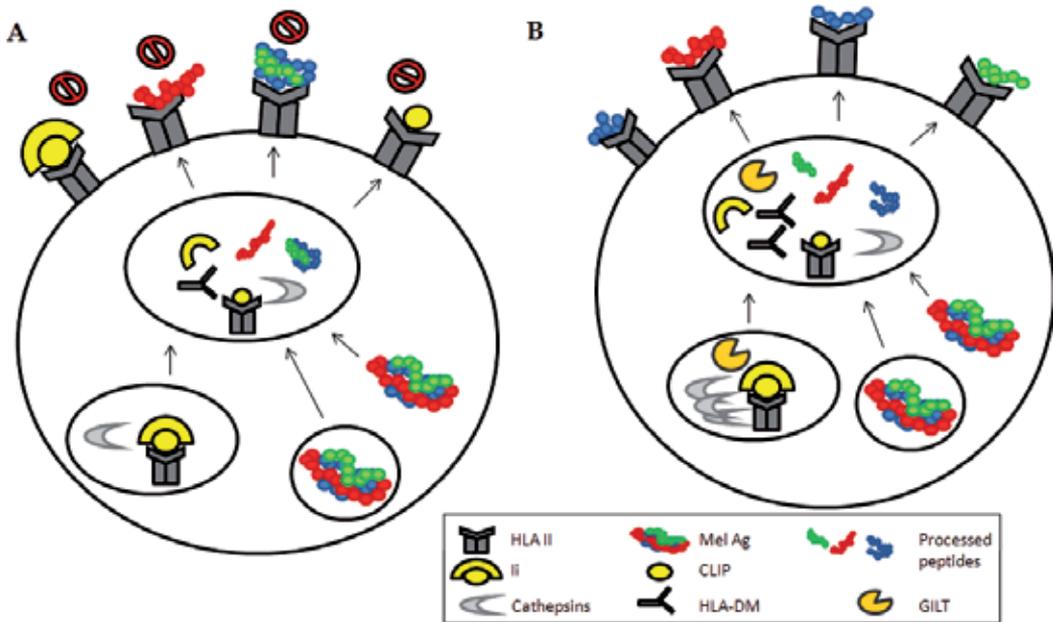


Fig. 2. Defects in HLA class II Ag Processing in Melanoma cells. A. Melanoma cells differentially process endogenous and exogenous Ags in endolysosomal compartments through deficiencies in Ii, HLA-DM, acidic cathepsin activity, and the failure to reduce oxidized peptides. This results in the presentation of a peptide milieu which fails to stimulate interacting CD4+ T cells, limiting the effects of CD8+ antitumor responses. B. The presence of GILT in endolysosomal compartments facilitates increase in both acidic cathepsin processing of tumor Ag and HLA class II components, and the functional processing of cysteinylated or oxidized peptides for improved CD4+ T cell activation.

Melanoma cells also differentially express acidic cathepsins which catalytically process endogenous and exogenous Ags in endolysosomal compartments. The lack of these enzymes or their limited activity results in poor Ag processing and the generation of nonfunctional antigenic determinant(s), that when presented are incapable of stimulating T cells (Goldstein et al., 2008). They also express low levels of HLA-DM, a nonclassical class II molecule responsible for peptide loading onto HLA class II molecules and the removal of the class II-associated invariant chain (Ii) peptide (CLIP) (Norton & Haque, 2009). Without active HLA-DM function, low affinity peptides are loaded onto class II proteins and in some cases CLIP is not removed, the result of which is again poor immune activation (Weber et al., 1996). Melanoma cells also lack an important enzyme, Gamma Interferon-inducible Lysosomal Thiol Reductase (GILT), which is required for the functional reduction of cysteinylated or oxidized proteins and peptides (Haque et al., 2002). Spontaneous cysteinylated peptides and Ags occurs through the formation of disulfide bonds between cysteine residues or when cysteine residues bind to free floating cysteine in biological fluid (Haque et al., 2002). These peptides display high binding affinity for the HLA class II binding groove, yet they are nonfunctional at CD4+ T cell activation. Figure 2 illustrates some of the defects in HLA class II processing and presentation utilized by melanoma tumors and the ability for GILT induction to improve or reverse some of these deficiencies.

GILT is a lysosomal reductase that is highly expressed in professional APCs, but is absent or expressed at low levels in melanoma (Phan et al., 2002). GILT can be induced in melanoma cells and in other tumor cells when treated with interferon-gamma (IFN- γ) (Goldstein et al., 2008). The expression of GILT in melanoma cells upregulates active forms of cysteinyl and aspartyl cathepsins such as cathepsins S, B, and D; and GILT expression also upregulates the non-classical class II molecule DM (Goldstein et al., 2008). GILT also breaks disulfide bonds within Ags/peptides providing further access for loading and processing by cathepsins (Goldstein et al., 2008). Melanoma cells expressing GILT may be able to efficiently process and present peptides to CD4⁺ T cells for immunological recognition and elimination of tumors. Unfortunately, the numerous defects illustrated here do not form the complete picture of melanoma immunomodulation in terms of Ag processing. Once functional peptides are loaded into either HLA class I or class II compartments and CD8⁺ and CD4⁺ T cells recognize these molecules, a second signal received from costimulatory molecules on the surface of the tumor is needed to activate these immune cells.

3.2 Costimulatory molecules

Surprisingly, melanoma tumors represent immunogenic cancers with various activation of antitumor immunity, yet the natural immune responses are incapable of eradicating the tumor, the reason for which is not fully understood (Pandolfi et al., 2008). A contributing factor to this is the previously mentioned immunosuppressive microenvironment and the altered Ag processing and presentation in melanoma that can stimulate T cells, but not in the same way as APCs which would drive a strong immune response against the tumor. A second factor influencing this inhibition is the lack of, or inhibition of, costimulatory signals required for optimum T cell activation. Following Ag processing and the loading of tumor derived peptides into the HLA class II groove, this complex is translocated to the cell surface for presentation to T cells. CD4⁺ T cells recognize functional class II complexes with antigenic peptides and tight junction binding occurs between the T cell receptor (TcR) and the class II/Ag complex (Cochran et al., 2000). CD4 molecules on T cells then bind to a different site on the HLA class II molecule and T cells receive their first stimulation signal (Chambers, 2001). A second signal is then required for activation/regulation of the T cell. If the T cells receive a stimulatory signal from the tumor in the form of CD80/CD86 (B7-1/2) binding to T cell expressed CD28, then T cells become activated and mount an antitumor response (Figure 3). However, costimulatory molecules are often modified on melanoma tumor cells inhibiting T cell activation. Melanoma tumors have been shown to express high levels of CTLA-4, a cell surface receptor that also interacts with CD28 but in a regulatory role, inhibiting T cell activation (Weber, 2008). Naturally, this function may inhibit autoimmune conditions, but tumors exploit this process, functionally silencing CD4⁺ T cell activation and shifting the environment to a T-regulatory setting.

These issues are further compounded by the presence of death receptor ligands on the surface of melanoma tumors (Pilon-Thomas et al., 2010). T cells naturally express programmed death receptors (PD-1) on their cell surface as a limiting factor during T cell activation, sparing healthy "self" cells that may activate these T cells. TIL's have been specifically shown to express higher levels of PD-1 than circulating T cells, a paradigm that is not completely understood (Ahmadzadeh et al., 2009). Study shows that melanoma tumors express high levels of the ligand for PD-1, PD-L, which during TcR-HLA interaction sends a death signal to both CD4⁺ and CD8⁺ T cells causing them to undergo apoptosis

(Pilon-Thomas et al., 2010). Melanoma specific-myeloid suppressor cells also express PD-L, further accelerating immunosuppression. Thus, these molecules represent ideal targets in developing improved melanoma immunotherapy strategies which will be discussed further in the following sections, particularly the use of anti-CTLA-4 monoclonal Ab (mAb).

An additional signal worth mentioning is the much debated role of tumor FasL (CD95) (Hallermalm et al., 2004). FasL is a transmembrane protein belonging to the TNF superfamily, which when bound to its receptor induces apoptosis (Hallermalm et al., 2004). Multiple studies have shown melanoma tumors express detectable surface FasL expression both *in vivo* and *in vitro* and that this ligand may act as a first line immunosuppressor through inhibiting CTL activity (Shukuwa et al., 2002; Andreola et al., 2002). High surface FasL expression also correlates with poor disease prognosis, but whether this is due to enhanced immune impairment or through an autocrine tolerization against FasL-FasR binding remains unknown (Hallermalm et al., 2004).

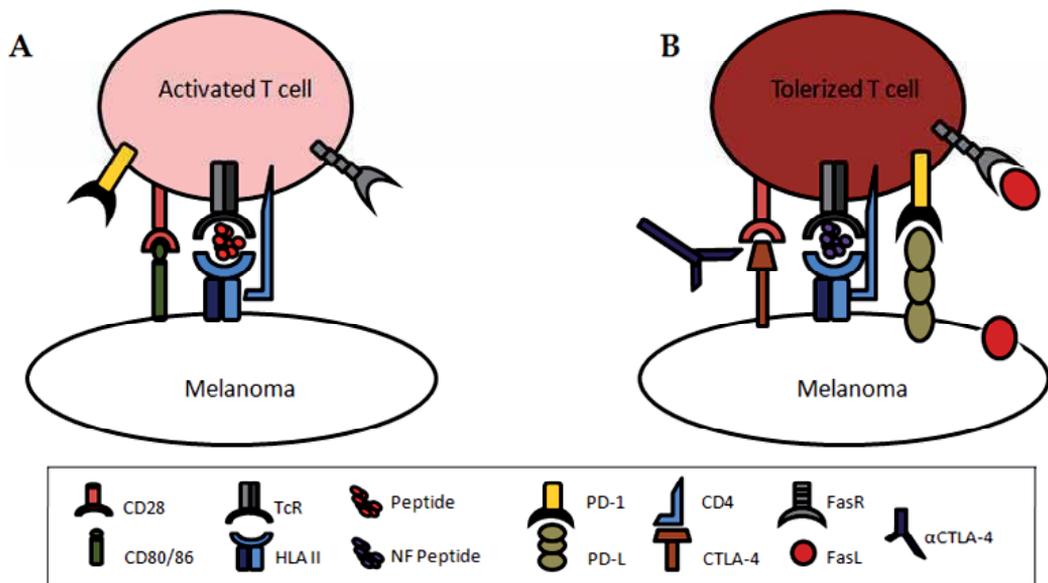


Fig. 3. Costimulatory Signals in Melanoma. A. Complete T cell activation requires binding between HLA class II-Ag complexes with T cell receptors on CD4⁺ T cells. A second signal between B7-1/2(CD80/86) molecules on melanoma tumors with CD28 on T cells activates CD4⁺ T cells initiating a robust immune response B. Melanoma cells differentially present oxidized peptides to the TcR which can inhibit T cell activation. They also express high levels of CTLA-4 which preferentially binds CD28 and suppresses T cell function. Blockade of CTLA-4 using mAb can restore immune recognition. Melanoma cells also express programmed death ligands (PD-L) which bind PD-1 on T cells inducing T cell tolerance. In addition, tumors also express surface and secretory FasL which bind FasR on T cells favoring apoptosis.

Study in uveal melanoma has also shown the potential for shed FasL encapsulated in a microvesicle that degranulates in the microenvironment and binds to FasR expression on lymphocytes (Andreola et al., 2002). More research is needed to fully develop this concept, but the targeting of surface FasL on melanoma tumor may improve the immune

environment allowing immunotherapy strategies to be more effective. More effective targeting of these factors is also paramount in limiting disease progression before tumors undergo angiogenesis and eventually metastasize, which pose their own issues in targeting the disease.

4. Issues with advanced disease

The previous sections have discussed the immunological concerns with melanoma in the tumor microenvironment and the interplay between the tumor and the immune system. However, the course of tumor progression further complicates this picture as tumors develop microvasculature, migrate to the blood stream, and metastasize to distant sites. Angiogenesis is tightly linked with the vertical growth phase of melanoma; a process which restructures the surrounding environment allowing tumor cells to migrate to the blood stream. This process is accompanied by a shift in the immune environment as infiltrating monocytes and macrophages converge at the site of angiogenesis and are misused by tumor cells to further proliferate. Following angiogenesis, melanoma tumors often metastasize to distant and local sites contributing to the great number of melanoma associated mortality. Once melanoma tumors invade the vasculature and colonize distant sites, new immunologic dysfunction is enacted at the site, further complicating and reducing the efficacy of therapeutic strategies, particularly in the lymph nodes and the brain. This section will further examine the immunological issues presented during angiogenesis and metastasis, and stress the need for new techniques at targeting these processes and how it will require specialized immunotherapy strategies.

4.1 Angiogenesis

As melanoma tumors progress, there is a distinct shift from the radial growth phase to the vertical growth phase which is accompanied by many changes to the cellular and immune environment. This change is also closely linked with angiogenesis. Angiogenesis is the formation of new blood vessels, and is abused and unregulated in advanced melanoma (Mahabeleshwar & Byzova, 2007). Figure 4A shows some of the molecules involved in angiogenesis, and in the switch from radial growth to vertical growth in melanoma tumors. With the formation of new blood vessels and the vertical growth of tumors, an influx of immune cells occurs as a result of shed molecules like IL-8 (Waugh & Wilson, 2008).

As previously discussed, IL-8 as well as IL-6 can act as circulating tumor cell attractants, which can accelerate tumor growth, angiogenesis, and recruitment of other chemo-attractants (Kim et al., 2009). IL-8 signaling has also been shown to increase the transcription of nuclear factor- κ B in melanoma, which may be increased through protein kinase C (PKC) activity (Wang & Richmond, 2001). Along with NF- κ B, STAT3, and β -catenin, IL-8 indirectly upregulates the activity of AP-1 and mTOR, which are both implicated in cell proliferation, invasion, and cell survival (Karst et al., 2009). VEGF is also linked with IL-8 signaling through its activation by the GPCR of the IL-8 receptor. VEGF is the primary molecule responsible for angiogenesis in both natural and melanoma settings (Srivastava et al., 2003). Melanoma cells have also been shown to utilize VEGF in an autocrine fashion to fuel progression and growth. Tumor-derived molecules like basic fibroblast growth factor (bFGF), placental growth factor (PGF), and platelet derived growth factor (PDGF) aid in melanoma angiogenesis, and may represent potential targets in immunotherapy design (Figure 4). Other angiogenic factors involved in melanoma are the matrix metalloproteinases

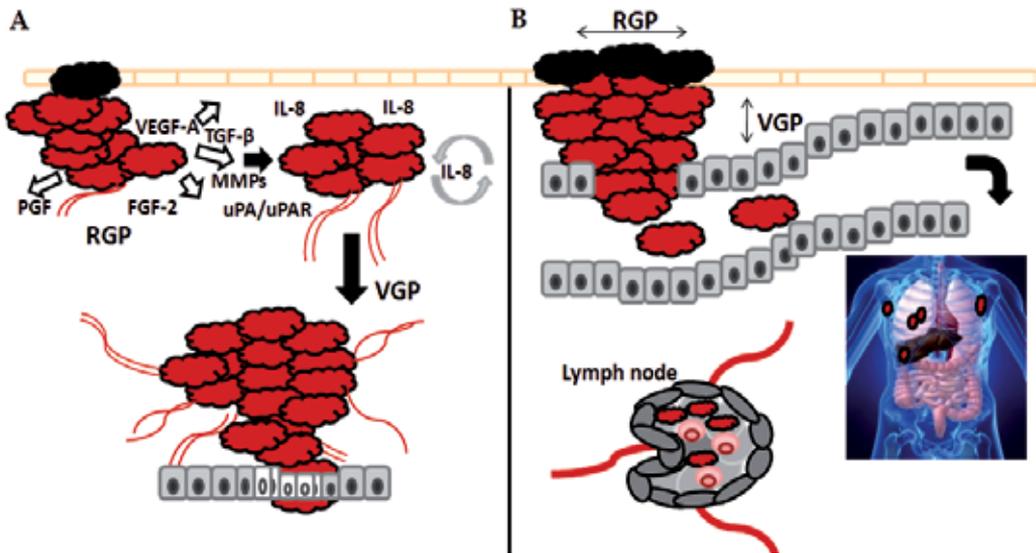


Fig. 4. Angiogenesis and Metastasis in Melanoma. A. Extracellular restructuring molecules are shed by both metastatic tumors and infiltrating bystander cells. These molecules (VEGF, PGF, FGF-2, and MMPs) lead to increased cell-cell contact, movement, and vascularization. The transition into vertical growth phase is then accompanied by increased autocrine IL-8 signaling which supports angiogenesis through recruitment of tissue destabilizing molecules and immune cells. Urokinase plasminogen activator and its receptor (uPA/uPAR) further angiogenesis by promoting cell movement and reorganization of endothelial cells into tube-like structure allowing tumors to reach the blood stream. B. Following RGP and VGP tumors metastasize to colonize distant sites such as sentinel lymph nodes, the lungs, liver and brain by entering the blood stream or through the lymphatic network. Within lymph nodes further immune suppression is induced through high levels of T regs, IL-10, and immature DC.

(MMPs). MMPs are a large group of secreted proteases that are involved in normal physiological and pathologic processes such as embryogenesis, wound healing, angiogenesis, tissue remodeling, tumor invasion, and metastasis (Kondratiev et al., 2008). Within this family of proteins, MMP-2, MMP-9, MMP-13, and MMP-14 have been found in melanoma, and are used as biomarkers for staging (Bosserhoff, 2006; Beshir et al., 2010). MMP-2 and MMP-9 degrade connective tissue and basement membrane collagen, and are believed to play an important role in skin and uveal melanoma progression (Kondratiev et al., 2008; Seftor et al., 2001). During the utilization of these molecules and others, there is localized inflammation occurring with the influx of lymphocytes, monocytes and macrophages. These cells are then activated and secrete TNF- α and IL-1 α in response to the shed VEGF and IL-8, which tumor cells then use in furthering their proliferation (Moldovan, 2002; Moldovan & Moldovan, 2005). In this system, the inhibition of macrophage/monocytes activation could aid in limiting tumor progression and angiogenesis. In an expert review of angiogenesis in melanoma, Ria et al describe the complete angiogenic process, the molecules involved, and the strategies designed to target these factors (Ria et al., 2010). The targeting of these molecules and the inhibition of the pro-tumorigenic influx of immune cells should aid in improving melanoma immunotherapy strategies.

4.2 Metastasis

Following melanoma angiogenesis and the vertical growth of melanoma into localized blood vessels, tumors metastasize to multiple distant sites including lymph nodes, the liver, lungs and brain (Streit & Detmar, 2003; Zbytek et al., 2008). This process has often occurred by the time melanoma is detected clinically, further complicating the treatment of the disease (Murakami et al., 2004). The first site of tumor metastasis is often the sentinel lymph node, which also serves as an important prognostic marker of melanoma progression (Takeuchi et al., 2004; Streit & Detmar, 2003). This progression to sentinel lymph nodes is closely related with angiogenesis, as these sites in metastatic melanoma patients have high expression of VEGF molecules (VEGF-C and VEGF-D) providing the link from tumor progression to metastasis. This process is also further driven by chemoattractive activity of tumor associated lymphatic endothelial cells which may act to draw melanoma cells to the lymph node (Kakinuma & Hwang, 2006; Streit & Detmar, 2003). This activity is a natural means of attracting mature dendritic cells to sentinel lymph nodes, but some melanoma tumors express the same surface receptor (CCR7) as dendritic cells, and are mistakenly drawn to the lymph nodes (Murakami et al., 2004). This is interesting, as targeting this surface receptor may aid in the prevention of melanoma metastasis.

The deregulated immune environment is also responsible for the spread of melanoma tumors from tissue to lymph nodes. The highly immunogenic nature of melanoma and the manipulation of immune cells by tumors, particularly in the development of Tregs, could contribute to the movement to sentinel lymph nodes. Within these lymph nodes, melanoma tumors further suppress the influx of anti-tumor immune cells through the tolerization of DC and the large number of Treg cells accompanying the tumor (Figure 4B) (Shu et al., 2006). As described in the immune cell section of this chapter, these Treg cells in the lymph are now capable of more widespread suppression allowing for the colonization of distant sites by travelling through the lymphoid network as veritable "body guards" surrounding the tumor. Tumors are also capable of traveling through the vascular network to colonize distant sites, which requires surviving the vascular environment, adhering to the desired organ, and invading the desired tissues. These processes are accomplished through upregulation of adhesion molecules on melanoma tumors like integrins allowing for adhesion and passage into distant tissue (Yoshimura et al., 2009).

Melanoma tumors now metastasize to distant sites including the liver, lungs, and brain. Single solitary tumors are effectively cleared through surgery, but multiple metastatic lesions limit surgeries effectiveness. The involvement of major organs represents a sharp decrease in the efficacy of treatment options, and with respect to brain metastasis, accounts for the overwhelming majority of melanoma deaths (Zbytek et al., 2008). Liver metastasis is often seen in cases of uveal melanoma and is treated through chemotherapy and surgery (Vahrmeijer et al., 2008). Unfortunately, unlike other cancers that metastasize to the liver, melanoma metastasis often cannot be resected due to the gross number of tumors and the location of the tumor (Vahrmeijer et al., 2008). Melanoma tumors which metastasize to the lungs often pose the same problems that liver metastases do, in which surgical resection is often impossible, and, to compound problems, brain metastases are often concurrent with lung metastasis, though the lungs are probably colonized first (Fidler et al., 1999). In these unique cases immunotherapy may be considered in place of or in conjunction with chemotherapy. A recent *in vitro* study on preferential liver metastasis of melanoma showed a correlation between the presence of high integrin 2 α on invading tumor cells versus those that did not metastasize to the liver (Yoshimura et al., 2009). Targeting this surface molecule

through the use of a monoclonal antibody could then prove useful in inhibiting disease before it progresses to the liver. Lung cancer can pose additional concerns; airway passage epithelial cells, like those found in the lungs, can possess TLR2 surface expression, which in vitro study has shown to be upregulated in metastatic melanoma cell lines (Yang et al., 2009). TLR2 activation can promote an influx of lymphocytes and cytokine production through STAT3 regulated pathways, which we have already mentioned, are deregulated in cases of melanoma. This same study showed that inhibiting TLR2 through the use of an anti-TLR2 antibody inhibited the extent of pulmonary melanoma metastasis, a concept which could prove useful in devising novel immunotherapy strategies (Yang et al., 2009). Despite brain metastasis resulting in the majority of melanoma deaths, relatively little is known about the processes involved in brain metastasis (Sloan et al., 2009). Patients with multiple melanoma brain lesions have few treatment options. When 1-3 brain metastases are present, surgery and stereotactic radiosurgery are routinely performed, but when more metastases are present surgery loses efficacy and response rates to chemotherapy remain low (Sloan et al., 2009; Aboody et al., 2006). Unfortunately, the presence of brain metastasis was often used as an exclusion criterion for immunotherapy trials of metastatic melanoma. Some studies using both biological response modifiers like IL-2/IFN α and cellular immunotherapy employing MART-reactive TIL's have shown some success (Sloan et al., 2009). Given the ability for some immune passage through the blood brain barrier, immunotherapy should be on the forefront of treatment for melanoma brain metastases, yet clinical manifestations of successful immunotherapy remain limited (Sloan et al., 2009).

5. Issues with immunotherapy

In many ways, melanoma represents the gold standard in immunotherapy design and clinical application. Melanoma is a highly immunogenic tumor, and clinical evidence has shown spontaneous regression of primary lesions in a significant number of tumors (Komenaka et al., 2004). This should not be confused with the outlined immunomodulation and immune deficiencies outlined in this chapter, melanoma tumors still evade immune clearance and detection, particularly in advanced metastatic disease. The study of melanoma immunotherapy has existed in some form for the last 30 years, with some major breakthroughs to show for all this hard work (Weber, 2011). In general, immunotherapy refers to any therapeutic intervention designed to stimulate, inhibit, support, or alter the immune system as a means of inducing tumor destruction. Immunotherapy strategies include the use of immunostimulatory cytokine administration like IL-2 and IFN α , adoptive cell transfer (ACT), multiple cancer vaccination strategies, and the use of monoclonal antibodies which target tumor Ag or suppress melanoma derived inhibitory signals (Weber, 2011). Some of the strategies used in a clinical setting are highlighted in Table 2. Surprisingly, with all of this work done, only recently has an immunotherapy shown a survival advantage in patients with advanced disease (Hodi et al., 2010). This section will briefly highlight some of the successes and issues with current immunotherapy, and try to determine how these deficiencies may be overcome. A more complete review of melanoma immunotherapy strategies can also be found from Sivendran et al (Sivendran et al., 2010).

The first immunotherapy strategies to show clinical promise were the immune stimulating cytokines IFN α and IL-2, both receiving FDA approval for the treatment of melanoma in the 1990's, IFN α in the treatment of stage III melanoma with Decarbazine and IL-2 in stage IV

Name	Action	Approval status	Clinical results	Issues
IFN-α (IFN- α 2 β , PEG-IFN)	Activates and stimulates DC and T cells, while cytotoxic to melanoma through STAT1 activation and STAT3 downregulation.	1995, As adjuvant with surgery or cytotoxic chemotherapy, most notably dacarbazine.	10-15% response rate, useful in cases of limited disease.	As single agent, high toxicity profile, adjuvant therapy has been linked with psychiatric issues like depression and mania.
IL-2 (Proleukin)	Expands and activates T cells, administered in bolus high dose.	1998, Stage IV melanoma.	15% response rate, some patients having durable responses to treatment, when in high dosage treatment regimen.	High toxicity profile. Best results only seen in a small inclusion group with limited disease progression and minimal organ involvement.
Canvaxin (onmelatucel-L)	Antigen-rich, allogeneic whole-cell vaccine.	Phase III trials ended in 2005 due to inefficacy.	Initial clinical trials were promising showing increased overall survival gains of 19 % and 12% in stage III and IV melanoma respectively.	During phase III clinical trials an independent safety reviewed board revealed Canvaxin was no more effective than control arm.
Adoptive cell therapy (ACT-CTL or ACT-TIL therapy)	Patient PBMCs are pulsed in vitro with melanoma peptide/Ag or stimulatory cytokines and CTL clones are isolated, expanded and injected.	Ongoing clinical trials using pretreatment of lymphodepletion through irradiation or chemotherapy prior to ACT injection.	Response rates have been reported as high as 50%, particularly in large bulk tumor cases when following lymphodepletion.	Lymphodepletion required for efficacy, but all patients can't tolerate.
Ontak (DAB/IL-2, Denileukin Diftitox)	Recombinant IL-2/ diphtheria toxin conjugate binds to IL-2R expressing cells depleting these lymphocytes.	Currently in Phase II clinical trial as intervention.	Promising results from phase II trials where lymphodepletion was observed with the formation of anti-melanoma CD8+ T cells.	While it depletes detrimental T reg cells, CD8+ and CD4+ effector cells are also depleted limiting overall efficacy.

Table 2. Issues Associated with Various Melanoma Immunotherapy Strategies

disease (Fang et al., 2008). IL-2 therapy remains the only immunotherapy strategy for late stage melanoma but significant issues remain with its use. IL-2 therapy displays moderate to severe toxicity and relatively low efficacy in patients with non-cutaneous metastasis and metastatic organ involvement (Petrella et al., 2007). Thus, the key to improving IL-2's use in a clinical setting is in the selection of patients receiving treatment. Younger patients who are in good health, with little to no organ metastasis would see the most benefit from IL-2 therapy, and patients with stage II/III tumors may see even greater response to this treatment. Similarly, IFN α displays toxicity similar to cytotoxic drug therapy and this toxicity increases greatly when administered over longer periods of time. Some study also showed a correlation between IFN α and clinical manifestations of depression, mania, and suicidal tendencies (Greenberg et al., 2000). The cause behind these effects is poorly understood, but will remain a concern to monitor with IFN α therapy moving forward. However, the main concern with IFN α is the lack of improvement in overall survival, with only transient gains seen in relapse-free survival (RFS) (Kirkwood et al., 2004). Efforts to combine IFN α with a cancer vaccine strategy were disappointing, but seem to reflect issues in the design and selection of the melanoma vaccine (Kirkwood et al., 2004). The use of IFN α

to boost an antitumor response paired with an effective vaccine or with ACT could still prove beneficial and shouldn't be ruled out in therapeutic design.

Another very promising concept in immunotherapy design which has failed to aid in the clinical setting is melanoma cancer vaccines. These can come in the form of whole cell cancer vaccines, tumor cell lysates, protein/peptide vaccines, DC loaded vaccines, viral vectors, and DNA vaccines (Terando et al., 2007). To date, the largest phase III melanoma vaccine clinical trial involving late stage III and IV melanoma compared the use of CanVaxin with the nonspecific immune stimulator Bacillus Calmette-Guerin (BCG) or BCG alone (Morton et al., 2002). BCG is currently being evaluated in phase III clinical trial administered following surgery versus best standard medical care alone in patients with advanced metastatic disease. CanVaxin is an allogeneic whole cell cancer vaccine using three of the most widely studied melanoma cell lines which encompassed a vast pool of Ag targets and showed great promise during phase II clinical trials.

Unfortunately, an independent safety review board halted the phase III trial when evidence showed no detectable advantage in the treatment arm, virtually stopping CanVaxin in its tracks (Eggermont, 2009). A key issue with the design and application of CanVaxin could be the lack of host Ag presented in the vaccine, given differences between the tumor cell lines used versus the primary tumor. Studies utilizing host-derived irradiated tumor cells instead of cell lines, have shown immune reactivity and limited toxicity, but the time required for their generation remains a concern. These cancer vaccine strategies have been investigated in early clinical trials but the clinical manifestation of strong antitumor T cell activation remains elusive (Goldman & DeFrancesco, 2009). Some efforts to improve the design and application of these vaccines will be further discussed in the next section.

Currently, an immunotherapy strategy with the potential for success is adoptive cell therapy (ACT). As previously mentioned, the highly immunogenic nature of melanoma tumors generates large pools of melanoma reactive CD8+ T cells in vivo, which can be extracted and expanded in vitro. This expansion of patient lymphocytes can be done through stimulation with T cell growth factors like IL-2 or through stimulation with melanoma tumor Ags (Rosenberg et al., 2003). Following expansion, these T cells are then re injected into patients to attack the tumor. This method has resulted in durable response rates, particularly in stage II/III patients; and in some cases, complete tumor reduction (Rosenberg et al., 2003). However, trials in late stage melanoma failed to show durable tumor clearance, most likely due to the high percentage of Treg cells and immunosuppressive cytokines in the microenvironment (Rosenberg et al., 2008). To combat this, the combination of lymphodepletion with chemotherapy or radiation prior to reinfusion of T cells can greatly improve the response to treatment (Dudley et al., 2008). Unfortunately, lymphodepletion in itself is hazardous to the patient as it destroys both the antitumor CD8+ and CD4+ effector cells along with the Treg cells, and leaves patients vulnerable to bacterial and viral infections (Dudley et al., 2008). Agents capable of specific Treg depletion would aid ACT therapy greatly, and a few molecules in clinical trials potentially fit this need. Ontak (Denileukin Diftitox), a recombinant fusion protein combining IL-2 with Diphtheria toxin, binds to IL-2R expressing cells and induces apoptosis through toxin release. It has been shown to deplete Treg cells, resulting in a CD8+ antitumor response in phase II clinical trials (Mahnke et al., 2007). However, CD4+ effector cells were also depleted following Ontak administration, a potentially limiting factor in the long term durable anti-tumor response. The combination then of Ontak with ACT could improve the efficacy of immunotherapy in advanced disease patients, but an important aspect of immune stimulation remains absent from this

therapeutic design: the activation of CD4⁺ effector T cells. As mentioned previously, CD4⁺ T cells play an important role in anti-tumor immunity particularly in the presentation of tumor Ags to professional APCs and CD8⁺ T cells, and in the induction of long-lasting anti-tumor immunological memory (Hung et al., 1998). Therefore, future strategies should aim to activate both CD4⁺ and CD8⁺ T cells in ACT therapy design, an idea which will be further explored in the following section.

6. Potential new directions

As highlighted in the previous section, our lack of success in melanoma immunotherapy is not for a lack of effort. With the last thirty years of immunotherapy design and clinical trials we can now apply what we've learned to novel ways of addressing the complex problem of metastatic melanoma. This may include revisiting some previously attempted ideas, but applied within a new context, particularly with what we know about the localized immune inhibition in melanoma patients. This section will discuss current strategies being developed and potential new directions in treatment that address the immunomodulatory nature of melanoma. These include novel chemoimmunotherapy ideas and techniques, the further use of monoclonal antibodies against melanoma Ags and T cell inhibitory factors and finally, on how combining multiple approaches in multimodal immunotherapy design represents a fight on multiple fronts with the potential for increased tumor destruction and disease free survival.

6.1 Chemoimmunotherapy

Therapeutic approaches combining cytotoxic chemotherapy with immunotherapy is not a new concept by any means (LoRusso et al., 1990). In fact, both IFN α and IL-2 have been extensively tested in combination with chemotherapeutics like decarbazine (DTIC), temozolamide, and cisplatin (Schadendorf et al., 2009). Yet, only the pegylated (PEG)-IFN α + DTIC or PEG-IFN α + temozolamide showed enhanced response rates, and all other trials failed to show any significant survival rates (Schadendorf et al., 2009). Moreover, the combination of these molecules increased overall toxicity greater than individual treatment, another limiting factor in the use of combined chemoimmunotherapy (Schadendorf et al., 2009). However, these results only indicate that the combination of two highly cytotoxic agents with limited efficacy in their own right are incapable of enhancing patient survival in highly advanced stages of diseases. Similar approaches should be carefully studied and considered in patients with diminished risk who demonstrate high tolerability to treatment, like the concession currently made for those receiving high dose IL-2 therapy. In selecting the right patient for this therapy, considerable gains may still be made in the combination of these agents. A second approach may be to limit the toxicity of the chemotherapeutic and select a drug with immunostimulatory properties. These molecules may include the highly en vogue antioxidant molecules like green tea extracts, holistic mushroom extracts, and flavinoids, each displaying cytotoxicity in tumor models *in vitro* with limited toxicity to healthy cells (Baliga & Katiyar, 2006; Harhaji Trajkovic et al., 2009; Craig, 1999). More importantly, these molecules may work synergistically with immune stimulating molecules through enhanced immune activation (Banerjee et al., 2008). Extensive research needs to be performed to ensure similar results *in vivo* as displayed *in vitro*, but the combination of these more tolerable antitumor agents with immunostimulatory cytokines may generate increased tumor clearance while reducing the toxic burden to individuals. Similarly, altering

the immune cytokine used could aid in the efficacy and reduced toxicity of chemoimmunotherapy.

Cytokines like IL-15, IL-7, and IL-21 could prove more beneficial in stimulating antitumor immune cells during chemotherapy, with reduced toxicity when compared to IL-2 or IFN α (Epardaud et al., 2008; Ribas, 2006). IL-21 has the added benefit of more selective T cell expansion versus IL-2, in which Tregs are not responsive to IL-21 stimulation (Sivendran et al., 2010). Currently IL-21 is being tested in phase I/II clinical trials displaying promising results (Sivendran et al., 2010). IL-15 shares similarity with IL-2 through a shared receptor subunit, but IL-15 has been shown to enhance both ACT and chemotherapy strategies in mouse models with almost no cytokine associated toxicity (Epardaud et al., 2008). Current research performed using IL-15 pre-conjugated with its receptor (IL-15 α) increased both the half life and activity of the cytokine, and improved its ability to destroy advanced solid tumors (Epardaud et al., 2008). Though preliminary, this discovery could prove beneficial in the use of cytokine therapy as both single agent, and in combination with chemotherapeutics to more effectively destroy melanoma. An additional strategy for combined chemoimmunotherapy use is in cancer vaccination design and application. In this case, chemotherapy prior to or concurrent with peptide or DC loaded vaccine techniques could allow for improved efficacy of a single treatment alone. Unfortunately, clinical trials have yet to show significant advantages to combining cytotoxic therapy with vaccine strategies over chemotherapy alone (Lens, 2008). To address this issue, study should incorporate drugs which target progression or metastasis of melanoma in conjunction with immunotherapy as a means of limiting tumor movement and increasing the chances of immune induced tumor clearance. As previously mentioned, Ria et al describe multiple anti-angiogenic drug candidates which could halt the progression of tumors through inhibiting VEGF, VEGFR, tyrosine kinase receptors, integrins, and MMPs (Ria et al., 2010). Combining these inhibitors with immune vaccines to stimulate antitumor immunity or with ACT could allow the reduction of both localized and metastatic tumors.

Currently, the most beneficial chemoimmunotherapy strategy uses lymphodepleting chemotherapeutics (cyclophosphamide and fludarabine) prior to reinjection with expanded TILs and high dose IL-2. This strategy vastly improves the response rate and efficacy of ACT through depletion of Tregs allowing for greater TIL expansion in vivo following reinjection. Unfortunately, lymphodepletion leaves the patient susceptible to both bacterial and viral infection, and is not specific for Tregs; all supportive anti-melanoma CD8 $^+$ and CD4 $^+$ T cells are also destroyed in the process. Treg-specific reduction prior to ACT represents the gold standard of improved treatment, yet as described previously our best efforts using drugs like Ontak are still shy of clinical relevance (Mahnke et al., 2007). Increased effort needs to be placed on developing Treg specific lymphodepleting chemotherapy, allowing for the full effect of ACT to show clinical benefit. These improvements to chemoimmunotherapy design are not the only potential new directions in melanoma therapy; the most promising option in the near future appears to be the use of monoclonal antibodies in the fight against advanced disease.

6.2 Monoclonal antibodies

Monoclonal antibodies (mAb) have been the focus of much research in cancer since their discovery some thirty plus years ago (Oldham & Dillman, 2008). Though some mAb have been approved to treat other cancers, most notably Trastuzumab (Herceptin) in the

treatment of breast cancer, there is only one FDA approved mAb for the treatment of advanced melanoma, ipilimumab (Dillman, 2011). Ipilimumab is one of a number of mAb designed to selectively block CTLA-4 activity in melanoma tumors allowing for the expansion and activation of CD8+ and CD4+ anti-melanoma T cells (Robert & Ghiringhelli, 2009). This chapter previously described the effect high CTLA-4 expression has on melanoma tumors by inhibiting the costimulatory signal needed for T cell activation, shifting the immune response from active to suppressive. Ipilimumab has been tested in clinical trials both as a single agent and in combination with chemotherapy and vaccine strategies (Wolchok et al., 2010; Weber et al., 2009). Recent phase III clinical trials of advanced disease showed, for the first time, a significant survival advantage of patients treated with ipilimumab versus a gp100 vaccine alone, representing a huge breakthrough in these advanced disease patients (Hodi et al., 2010). A larger phase III comparison study using DTIC ± ipilimumab involving more than 500 patients has been performed, the results of which led to the approval of Ipilimumab in the treatment of advanced melanoma on March 25, 2011. Ipilimumab, like any other treatment, is not without issue, as patients undergoing treatment have experienced immune-induced side effects including dermatitis, enterocolitis, hepatitis, and most significantly diarrhea (Sivendran et al., 2010). Some interesting treatment effects have also been observed in a number of patients treated with ipilimumab which will require close attention in determining the best treatment protocol. A number of patients receiving ipilimumab displayed delayed slow ongoing responses lasting a year or more, delayed responses taking up to 6-12 months, and surprisingly, tumor growth and progression followed by tumor regression (Weber, 2011). These interesting effects could lead to the withdrawal from treatment due to the appearance of tumor growth or the delayed response, but these patients need to remain in treatment as the patients who displayed these characteristics often had better disease prognosis than others (Weber, 2011). New immune criteria for the judgment of drug efficacy should be included in determining the best course of patient treatment to avoid issues like these, particularly as ipilimumab has great potential as a combined agent with vaccination and cytotoxic drug therapy, and most interestingly with ACT.

Ipilimumab is not the only mAb targeting advanced disease receiving clinical attention; anti-PD-1, anti-CD137, and anti-CD40 are all undergoing clinical investigation (Sivendran et al., 2010). Anti-PD-1 has shown promise in melanoma cell lines and small phase I/II clinical trials displayed immune activity without major drug associated toxicity, opening the door for its use in combination with other mAb and immunotherapeutic strategies (Brahmer et al., 2010). Clinical trials are ongoing, pairing anti-PD-1 with CTLA-4 blockade or with multiple melanoma peptide vaccine strategies (Curran et al., 2010). Anti-CD137 binds to 4-1BB expressed on activated immune cells sending a costimulatory signal promoting lymphocyte activation (Meseck et al., 2011). In trials using anti-CD137 with GM-CSF-secreting tumor cell immunotherapy induced complete rejection of tumor in a B16 mouse model, displaying proof of principal in melanoma immunotherapy use (Li et al., 2007). Results of a phase I clinical trial revealed antibody activity associated with minimal toxicity in patients with advanced solid-tumor malignancies (Sivendran et al., 2010).

Similarly, anti-CD40 binding with CD40 on the surface of immune cells results in the activation of T cells, and the upregulation of MHC class II complexes and costimulatory molecules, two characteristics we have previously described as being major roadblocks to immunotherapy of advanced melanoma (Law & Grewal, 2009). Anti-CD40 has been tested in advanced solid tumors, refractory Hodgkin lymphoma, and multiple myeloma (French et

al., 1999). In melanoma, anti-CD40 seems ideal for combination with protein/peptide vaccine strategies as upregulation of MHC class II complexes and costimulatory molecules would increase the amount of tumor-vaccine interaction and presentation to T cells where increased costimulatory molecules would promote T cell activation instead of suppression or T cell anergy. Future efforts in melanoma mAb design should include the targeting of immunosuppressive cytokines and molecules like those outlined at the start of the chapter as a novel mechanism of enhanced immune activation. These molecules include, but are not limited to, IL-10, TGF- β , PGE2, IDO, and VEGF. Through the targeting and inhibition of these molecules a more immunosupportive tumor microenvironment could increase the efficacy of current and future immunotherapy strategies. Along with cytokine therapy, the use of mAbs in the treatment of metastatic melanoma appear to have the fastest course to clinical use, but the ideal immunotherapeutic strategy would combine these techniques with ACT or vaccination strategies, promoting long-term, sustained immunological anti-tumor responses.

6.3 Multimodal therapy strategies

As outlined in this chapter, melanoma tumors deploy multiple immune evasion techniques to subvert both natural and therapy induced anti-melanoma immune responses, despite the highly immunogenic nature of melanoma tumors. It should then make logical sense that targeting one of these pathways or suppressive mechanisms would incompletely abrogate the problem, as different mechanisms would take over the immunomodulatory duties of melanoma tumors inhibiting the treatments efficacy. Thus, strategies to target melanoma should target multiple suppressive pathways preventing tumors from avoiding single agent strategies. This is accomplished by the administration of multimodal therapy design, in which the combination of therapies enhances the effectiveness of each individual therapy increasing the durability and intensity of the anti-tumor response (Kudo-Saito et al., 2005). Multiple *in vitro* and melanoma mouse models support the notion of multimodal therapy as a way to completely ablate tumor burden and prevent its recurrence (Ascierto et al., 2010). Clinically, the combination of strategies is not a new method by any means, and even current immunotherapy techniques often combine chemotherapeutic agents with immune cytokines and mAbs for improved treatment (Bhatia et al., 2009). However, in moving forward in the design of improved melanoma therapy, combining multiple immunotherapy strategies like, ACT, gene therapy, DC or peptide vaccines, cytokine therapy, mAb therapy, and novel tumor molecule targeting could represent the future standard of advanced melanoma therapy. Before this ideal can be realized, there are some issues with each technique individually that need to be fixed prior to their combination. Some of these issues we have already mentioned: the inherent toxicity of immune stimulatory cytokine like IL-2 and IFN α , the failures of ACT to fully stimulate long-term anti-tumor immunity, the time required in generating an expanded pool of TIL for ACT therapy, and the inefficacy of vaccine strategies *in vivo* like those seen with CanVaxin (Berinstein, 2009). The first two issues we have already addressed; the use of less toxic cytokines like IL-7, IL-15/IL-15R, and IL-21 could alleviate toxicity concerns while promoting the expansion of activated immune cells, particularly those which promote immunological memory, and through lymphodepletion prior to ACT expanded lymphocytes are capable of repopulating the immune environment with anti-tumor CD8 $^+$ T cells leading to longer efficacy (Dudley et al., 2008). The last two issues are more difficult to solve, but there is hope on the horizon.

The effect that melanoma has on the immune system both hinders our ability to treat the disease, and also allows for treatment like ACT to effectively kill tumors due to the immunogenic nature of melanoma. Unfortunately, only about 50% of melanoma patients have TILs capable of being expanded in vitro for reinjection, a number still much higher than in other cancers where the feat is near impossible (Rosenberg et al., 2008). Further complicating things, the generation of expanded activated TILs takes a few weeks, during which, valuable treatment time is lost for those patients with advanced disease, and with lymphodepletion the chances of an infection increase greatly (Rosenberg et al., 2003). Thus, the ability to expand activated patient TILs from all patients and the rapid generation of anti-tumor lymphocytes would further the effectiveness and clinical application of ACT (Rosenberg, 2001). To achieve this, rapid selection of CD8+ T cells with characteristics predetermined beneficial for anti-tumor immunity can be separated in culture and expanded with administration of anti-CD3 and the presence of feeder cells to increase the activation and expansion rate. Results from this treatment manipulation have shown benefit over previous strategies (Rosenberg et al., 2008). To combat the inability of all patients to generate active TIL, strategies which clone TcRs from activated TIL population into peripheral blood mononuclear cells (PBMCs) of melanoma patients allows for the reinjection of primed anti-tumor lymphocytes without the need for expanded TILs (Weber, 2011). An additional benefit of cloning in these TcRs is that they can be manufactured to express theoretically any melanoma Ag capable of synthetic production, improving the chances of T cell stimulation in vivo through cross-presentation (Robbins et al., 2011). Study using this method has shown benefit in a number of cancers, even in the absence of prior lymphodepletion, further reducing treatment associated toxicity (Robbins et al., 2011).

An additional strategy which should be explored is the expansion of CD4+ effector TIL in the same fashion as CD8+ CTL for combined ACT capable of a more prolonged interaction resulting in increased tumor clearance. These CD4+ TIL can be expanded and raised against patient derived or synthetically generated melanoma Ag, allowing for direct cross-presentation with patient lymphocytes in vivo. This could then lead to the development and support of CD8+ memory T cells, as long as the synthetic Ag is highly immunogenic and replicates the immunodominant HLA matched tumor epitopes. This last point, the ability to present synthesized melanoma Ag capable of stimulating patient immune cells in vivo is also a major issue in vaccine development. As previously mentioned, melanoma vaccine strategies are as varied as they are disappointing in the clinical setting facing issues of inefficacy, inferiority to chemotherapy, and a failure to work in collaboration with other techniques (Berinstein, 2009). Two key issues face melanoma vaccine strategies, the selection of immunogenic tumor associated Ags and the reliance on only CD8+ CTL stimulation in vaccine design, and in many ways these two problems go hand in hand (Sondak et al., 2006). In this chapter, we have highlighted the importance of stimulating CD4+ effector T cells in developing a robust immune response through cross-presentation to CD8+ T cells and other APC, but also for their release of immunostimulatory cytokines like IFN γ (Corthay et al., 2005). Yet, until recently, vaccine strategies (protein/peptide, whole cell, or DC loaded vaccines) have solely used tumor Ags designed for CD8+ CTL activation (Terando et al., 2007). Though these T cells are responsible for the bulk of tumor destruction, time and time again they fail clinically to eradicate host tumors, often due to induced T cell tolerance against these Ags propagated by tumors (Terando et al., 2007). Only through the discovery and application of HLA class II specific anti-tumor melanoma Ags, effector CD4+ T cells may get the much needed stimulation required for a complete T cell response.

Excellent work has been done in the field of HLA class II specific melanoma Ags in recent years, specifically in the identification of an HLA-DR4 restricted Melan-A/Mart-1₅₁₋₇₃ epitope (RNGYRALMDKSLHVGTCALTRR) capable of stimulating CD4⁺ anti-tumor T cells in a number of DR matched patients (Zarour et al., 2000). The identification of Ags like these should be added to patient specific vaccine strategies matching both HLA class I and HLA class II allele specific immunogenic Ags in order to improve the efficacy of melanoma tumor vaccines. A second vaccine strategy which stimulates both CD8⁺ and CD4⁺ T cells utilized a natural process of HLA class II peptide presentation in a whole cell cancer vaccine design for enhanced Ag-T cell interaction (Bosch et al., 2010; Thompson et al., 2008). This class II vaccine uses tumor cells genetically modified to express surface class II and costimulatory molecules without expressing invariant chain (Ii). As previously described, Ii blocks the loading of antigenic peptides into the HLA class II peptide binding groove prior to presentation in the endolysosomal compartments (Thompson et al., 2008). By silencing Ii, numerous novel class II peptides are loaded onto the HLA binding groove which would have normally been blocked (Thompson et al., 2008). These peptides may or may not be functionally active, but they do represent peptides which T cells are not tolerant against, allowing for potential T cell activation. The presence of costimulatory molecules on the vaccine-cell surfaces furthers the chance of immune activation, as T cells receive the support signal needed for activation. A third way to generate novel immunogenic peptides for HLA class II presentation, which has been briefly stated, is the induction of GILT into whole cell vaccine strategies. GILT has been shown to increase HLA-DM expression, cysteinyl protease activity, and functionally reduces cysteinylated or oxidized Ags/peptides when transfected into melanoma cells (Goldstein et al., 2008; Rausch et al., 2010). In much the same fashion as the previously described vaccine design, GILT's presence could generate novel immunogenic tumor Ags capable of stimulating CD4⁺ T cells in vivo. Though early in discovery, techniques like these should be pursued in the improved design of melanoma cancer vaccines. The combination of these improved techniques, with those already described, in a multimodal immunotherapeutic design could then provide the most benefit in terms of long-term tumor free survival. A proposed outline of a multimodal immunotherapy regime is shown in Figure 5.

By first clearing the Tregs from the tumor microenvironment, any immunotherapy strategy will see improved efficacy, similar to those seen in ACT clinical trials (Dudley et al., 2008). ACT TIL therapy utilizing both CD4⁺ and CD8⁺ activated and expanded T cells or through the addition of TcR-Ag specific transfected PBMCs are then free to repopulate the immune environment. TIL responses against the tumor should then sustain for much longer than previously observed with the addition of activated CD4⁺ T cells using HLA class II specific tumor Ags and with concurrent stimulation with mAb against CTLA-4. The patient should then be monitored for response to treatment seen in tumor reduction and by extracting a sample of patient TILs checked for tumor reactivity. The addition of an improved DC loaded vaccine pulsed with processed tumor peptide-GM-CSF fusion protein, like those used in the successful prostate cancer therapeutic Sipuleucel-T (Provenge), could then act as a boost to the anti-tumor TILs present sustaining the development of immunological memory (Brower, 2010). Finally, using less toxic cytokine therapy through IL-15/IL-15R or with IL-7, IL-21 will support the continuation of CD8⁺ memory T cells and fuel the remaining anti-tumor lymphocytes into attacking the tumor. Though complex, the combination of these procedures would more effectively target the multiple

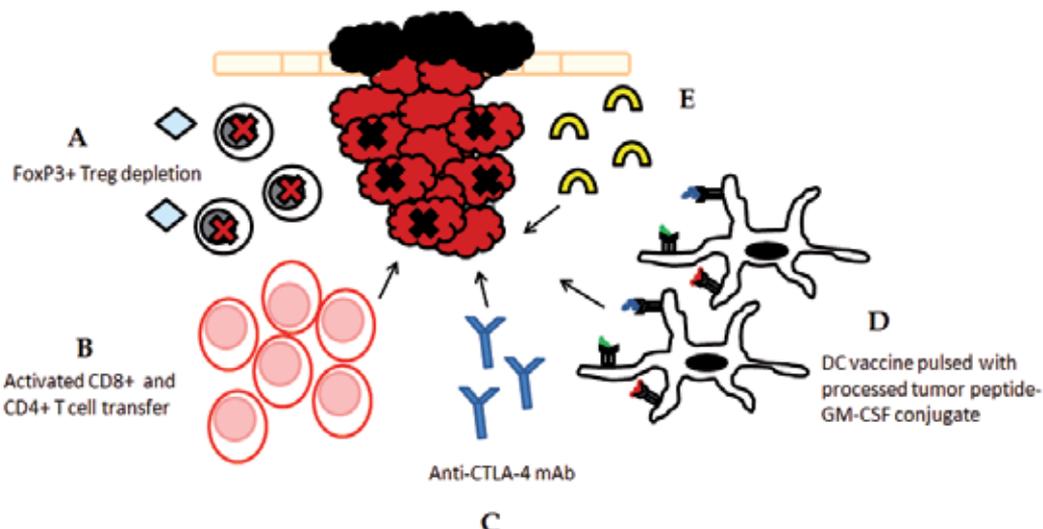


Fig. 5. Multimodal Therapy Design. A. Most of not all immunotherapy strategies should include a means of Treg depletion to prime antitumor immune responses. B. This may come in the form of an adoptive cell therapy (ACT) using autologous TIL's expanded in vitro and pulsed with tumor specific peptides targeting both CD8+ and CD4+ T cells. C. The addition of anti-CTLA-4 mAb could further support these TIL's life and prevent de novo generation of Treg cells D. Following a resting period to limit the chances of autoimmune reactions, concomitant challenge with a dendritic cell vaccine loaded processed tumor peptide and GM-CSF could then further expand the tumor specific immune response of the ACT. E. IL-15a therapy could also be administered during vaccination protocol to stimulate and expand T cell responsive with significantly less toxicity than through IL-2 addition.

immunomodulatory mechanism employed by tumors to evade the immune system. Much work will be needed to bring these theoretical ideals into clinical reality, but through improved understanding of melanoma tumors and their ability to modulate the immune environment, substantial gains can be made in both therapeutic design and patient care.

7. Conclusions

The future looks promising for melanoma immunotherapy, even with the multiple disadvantages that research scientists and clinicians face from the disease. This chapter attempted to summarize the multiple steps of immunomodulation that melanoma tumors employ to thwart even the most well thought out therapy strategies. By understanding both the mechanisms tumors employ to suppress and abuse the immune system and the ways that previous immunotherapy strategies have failed to meet our expectation, we can move forward in devising new strategies in improved immunotherapeutics and disease management. These improved concepts include combining chemotherapy and immunotherapy strategies in new ways to more effectively shift the balance of the tumor microenvironment from immunosuppressive to immunostimulatory prior to immunological intervention, and the targeting of protumorigenic pathways like the BRAF/MAPK and STAT3 pathways to aid in tumor destruction and promote immune responses. Improved understanding of the defective Ag processing and presentation pathways in melanoma

presents novel targeting strategies through genetic manipulation of tumors by induction of GILT capable of generating novel immunogenic Ags/peptides. The importance of incorporating strategies to expand and activate both CD8+ and CD4+ T cells will also help in the design and efficacy of ACT, whole-cell, and protein/peptide based vaccine therapy with improved durable immune responses and the development of immunological memory. New cytokine adjuvants like IL-15 and IL-7 may also expand the efficacy of these techniques and aid in the reduction of toxicity seen in IL-2 or IFN α strategies. Monoclonal antibodies like ipilimumab also represent the very near future in melanoma therapy following its recent FDA approval. More strategies combining these molecules with ACT and vaccine strategies should be explored as a means of enhancing the expansion and activation of immune cells and in avoiding immunosuppression induced by melanoma cells. More collaborative efforts need to be made between basic scientists, clinical investigators, and physicians to devise the best way to take discoveries made on the bench into the clinic, particularly in the immunomodulation of melanoma tumors. It appears that for every step we take forward in targeting melanoma, the tumors develop a new suppressive mechanism to avoid our efforts. These deficits must be understood and overcome for immunotherapy to become a true therapeutic option for late stage melanoma patients. As always our best weapon in the fight against melanoma is knowledge, and the collective knowledge gained from understanding melanoma immunomodulation will lead to the next generation of techniques capable of alleviating this deadly disease.

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Simultaneous Knockdown of Mutant BRAF and Expression of INK4A in Melanoma Cells Leads to Potent Growth Inhibition and Apoptosis

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1. Introduction

Melanoma is the eighth most common cancer in the United States and incidence is increasing at a rate greater than all other cancers. Estimates indicate that in 2010 there were 68,130 new cases of melanoma in the United States and 8,700 deaths from the disease. Overall, the lifetime risk of developing melanoma is about 2% (1 in 50) for Caucasians, 0.5% (1 in 200) for Hispanics, and 0.1% (1 in 1,000) for African Americans (American Cancer Society). Malignant melanoma results from transformation of melanocytes with progression through histologically recognizable sequential steps including radial growth phase (RGP), vertical growth phase (VGP), and metastasis (Elder, 1999). In RGP, neoplastic cells are confined to the epidermis or with microinvasion into the dermis. In more advanced VGP, cancer cells expand in the dermis and generate tumor nodules and have a high potential for metastatic spread. In the metastatic phase, cancer cells disseminate to lymph nodes or distant organs (Clark & Tucker, 1998; Elder, 1999). Optimal treatment varies based on stage of disease. For the early diagnosed and localized melanomas, surgery is the treatment of choice and may be curative provided the lesion is excised completely. However, for invasive and metastatic melanomas, there is no effective treatment and many patients die within 6-8 months after diagnosis. The aggressive growth of melanoma cells and their intrinsic resistance to standard modalities of cancer treatment account for the dismal prognosis (Poulikakos & Rosen, 2011; Tawbi & Buch, 2010). Therefore, the ability to inhibit growth and overcome drug resistance is central to the effective treatment of melanomas.

Treatment options for advanced melanoma are currently limited to immunotherapy, single agent cytotoxic chemotherapies, and surgery. Recently, Yervoy (Ipilimumab), an inhibitor of cytotoxic T-lymphocyte antigen 4 (CTLA-4) gained Food and Drug Administration (FDA) approval to treat metastatic melanomas. It is the first drug shown to prolong the lives of patients with advanced melanoma (Weber, 2011). However, its effect is limited. Patients with metastatic melanoma treated with Yervoy lived a median of approximately 10 months compared with 6.4 months for patients in a control group. Treatment with interferon Alpha 2B has shown improvement in disease free survival although overall survival benefit results are conflicting (American Cancer Society, <http://www.cancer.org/>). Interleukin 2 has also shown activity against metastatic melanoma although patient populations are limited by severe toxicity. Dacarbazine is the most studied of single agent cytotoxic agents for

malignant melanoma and is considered gold standard for comparison of systemic chemotherapy. However, no survival benefit has been shown in randomized controlled trials. Complete response rates are 3-5%, and long term remission rates are less than 2% under systemic treatments for metastatic cutaneous melanoma. Combination chemotherapy involving dacarbazine increases toxicity without improvement in median survival compared to dacarbazine alone. Metastatic melanoma can sometimes be treated with surgery. Patients can achieve prolonged overall survival after complete resection of tumors. Unfortunately, beneficiary outcome of surgical resection is limited to single or solitary melanoma metastases compared with patients with disseminated disease (Sosman et al., 2011).

Results of the recent phase I clinical trial with PLX4032, a specific inhibitor of mutant BRAF, have generated great excitement because approximately 80% of BRAF mutant metastatic melanomas regressed in response to PLX4032 treatment. Though this trial was considered a true victory in the fight against melanomas, attention has been drawn to the fact that regressed tumors resurge more aggressively within 8 months after the start of therapy. Constitutive deregulation of BRAF-MEK-ERK and p16-CDK4-RB pathways occur at high frequencies in melanomas. We have shown that suppression of either BRAF-MEK or CDK4 activity inhibits cell growth, and that simultaneous inhibition of both BRAF-MEK and CDK4 activities compounds this effect and triggers significant apoptosis in melanoma cells. Our data suggest BRAF-MEK-ERK and p16-CDK4-RB pathways may act additively or synergistically in the malignant growth of melanoma cells, and could be jointly targeted for treatment of melanoma. We have recently reported that the expression of K type human endogenous retrovirus (HERV-K) correlates with ERK activation and p16 loss in melanoma cells and can be inhibited by MEK and CDK4 inhibitors, especially in combination. Given that HERV-K may drive malignant growth downstream of BRAF-MEK and CDK4, we hypothesize cells with activated HERV-K may escape the therapeutic effects of MEK and CDK4 blockers, and that triple inhibition of BRAF-MEK, CDK4, and HERV-K should be more effective than single or double inhibition.

2. Mutational activation of NRAS-BRAF-MEK-ERK pathway in melanoma cells

In a systematic genome-wide screen for gene mutations, Davies et al. (Davies et al., 2002) identified BRAF mutations in 70% of human malignant melanomas. A T1799A transversion in exon 15, resulting in a V600E substitution in the BRAF kinase domain, accounts for over 90% of BRAF mutations detected in melanoma samples. In addition to melanomas, BRAF mutations have been identified in several other tumor types including thyroid, ovarian, colorectal, and lung (Davies et al., 2002). BRAF, one of three members of the RAF family (ARAF, BRAF, CRAF), is a component of the RAS-RAF-mitogen activated protein kinase/ERK kinase (MEK)-extracellular signal regulated kinase (ERK) signaling pathway. RAF gene encodes cytoplasmic serine/threonine kinases that transduces regulatory signals from RAS to MEK-ERK (Mercer & Pritchard, 2003). RAF genes are differentially expressed with CRAF being ubiquitously expressed; ARAF predominantly found in urogenital tissue, and BRAF having highest expression in neural and testes (Mercer & Pritchard, 2003). Mutant BRAF has increased kinase activity causing intrinsic ERK activation in cultured NIH3T3, COS, and human melanocytes, and leads to elevated transforming activity of cultured NIH3T3 and human melanocytes (Davies et al., 2002; Dong et al., 2003; Satyamoorthy et al., 2003; Wellbrock et al., 2004). Suppression of mutant BRAF expression

has been reported to cause inhibition of melanoma cell proliferation and survival *in vitro* and *in vivo* (Hingorani et al., 2003; Karasarides et al., 2004; Rotolo et al., 2005; Sumimoto et al., 2004).

In addition to BRAF mutation, NRAS mutation occurs in about 10% of melanomas (Davies et al., 2002; Dong et al., 2003; Michaloglou et al., 2008) that can also lead to constitutive activation of the MEK-ERK pathway. Apart from NRAS and BRAF mutation, other factors have been identified leading to constitutive activation of the ERK signaling. For example, amplification and somatic mutations of KIT and constitutive expression of growth factors HGF and FGF (Fecher et al., 2008; Panka et al., 2006a). The RAF-MEK-ERK pathway conveys extra- and intracellular signals to transcription regulators that control gene expression in response to these signals. The pathway impinges on all the functional hallmarks of cancer cells including immortalization, growth factor independent proliferation, loss of responsiveness to cell cycle checkpoint signals, evasion of apoptosis; insensitivity to growth inhibitory signals, ability to invade and metastasize, and ability to attract blood vessels (Chang et al., 2003; Kolch, 2000; Pearson et al., 2001) and represents rich druggable targets for drug development (Fig. 1, <http://clinicaltrials.gov/>) (Fecher et al., 2008; Panka et al., 2006a).

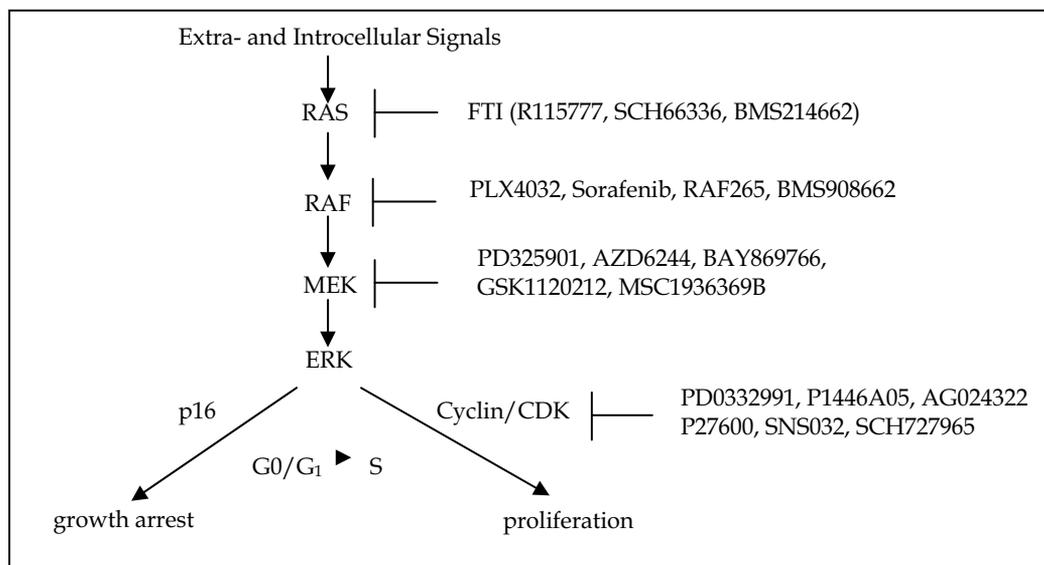


Fig. 1. RAS-RAF-MEK-ERK signaling pathway and specific inhibitors. RAF relays RAS signals through MEK to ERK. The activation of this pathway has multiple effects on cell proliferation, differentiation, and survival depending on the cellular contexts. Also shown are inhibitors to components of the pathway that are in active clinical trials (<http://clinicaltrials.gov/>). FTI, farnesyl transferase inhibitor.

3. Detection and specific inhibition of mutant BRAF

We performed research and validation studies to detect BRAF codon 600 mutations (Dong et al., 2003; Rotolo et al., 2005). As shown in Figure 2, a PCR and Sanger sequencing assay is used to detect BRAF codon 600 mutations. The common T1799A transversion changes wild-type GTG (valine) to GAG (glutamate) at codon 600 (V600E). In addition, a two-nucleotide

substitution, GTG to AAG (lysine), V600K, is occasionally identified in melanoma cells (Fig. 2A; Dong, 2003). BRAF proteins harboring V600E or V600K mutations, but not wild-type BRAF, are specifically inhibited by mutant specific BRAF inhibitor PLX4032 (Bollag et al., 2010; Flaherty et al., 2010; Rubinstein et al., 2010; Solit & Rosen, 2011). It is interesting to note that although most BRAF mutant alleles co-exist with wild-type copies, some melanoma cells show loss of the wild-type allele (Fig. 2A, A1799 LOH, AA1798-1799 LOH). Although rare, these cases suggest that loss of wild-type allele may offer growth advantage to cancer cells. We developed a PCR and restriction fragment length polymorphism (RFLP) assay (PCR-RFLP) to detect the common T1799A BRAF mutation (Fig. 2B). We designed a PCR forward primer (5'-GTA AAA ATA GGT GAT TTT GGT CTA GCT GAA G-3') to create, when the mutant A1799 follows in the DNA template, an MboII restriction site [GAAGA(N8)↓]. The reverse primer also has an MboII site for enzyme digestion and size control. PCR products are digested with MboII and restriction fragments separated by electrophoresis (Fig. 2B). The method is validated by direct sequencing of PCR products. Using this method, we identified melanoma cell lines that are wild-type (1363Mel), heterozygous (624Mel, WM35) or show LOH (A375) for the T1796A mutation (Rotolo et al., 2005).

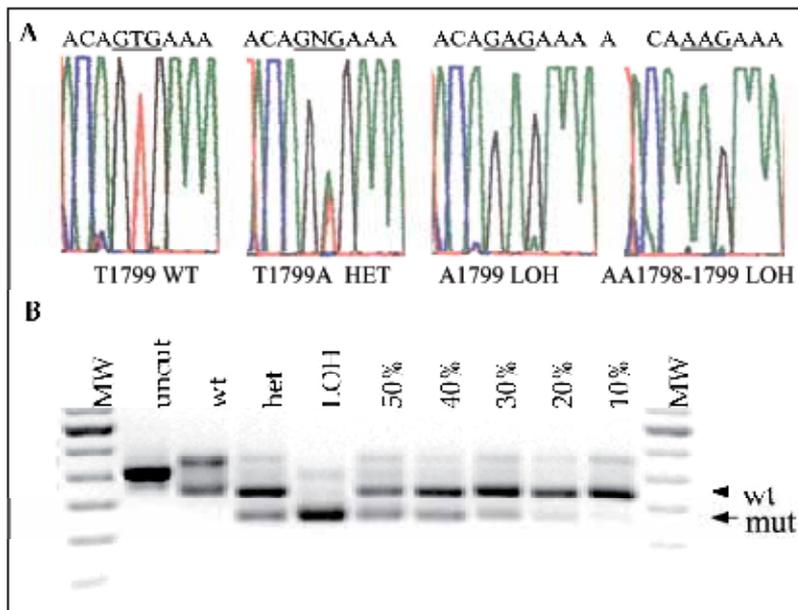


Fig. 2. BRAF codon 600 mutation analysis. (A) Wild-type (wt) and mutant (mut) alleles are detected by Sanger sequencing. The heterozygous (HET) sequence showed approximately 50:50 ratio of wt vs. mut alleles. Samples with no detectable wild-type allele are called loss of heterozygosity (LOH). A two-nucleotide substitution, GTG to AAG, is occasionally identified in melanoma specimens. (B) PCR-RFLP assay to increase sensitivity of detecting mutant allele (10% vs 30% by Sanger sequencing). Control and enzyme digested PCR amplicons are separated by electrophoresis on a 3% agarose or 10% polyacrylamide gel. The undigested PCR amplicon, digested wt and mut bands are 255, 218, and 178 bps, respectively. Serial dilutions containing 50%, 40%, 30%, 20%, and 10% of mutant alleles are used to determine assay sensitivity. Various heteroduplex bands are visible. MW, 50 bp molecular weight marker.

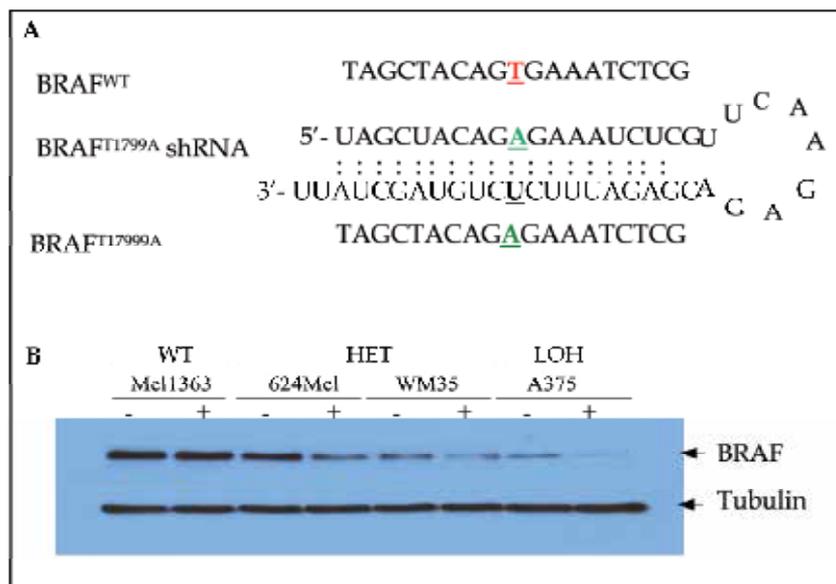


Fig. 3. T1799A mutant specific RNAi. Control and mut BRAF RNAi retroviruses are produced to infect melanoma cell lines. (A) The predicted BRAF^{T1796A} shRNA transcript encoded by pRS-BRAF^{T1799A}. Shown are sequences of the wt and T1799A mut alleles, and the predicted shRNA. (B) Western blot analysis of specific inhibition of mut BRAF by RNAi. Melanoma cells are stably infected with control (-) or pRS-BRAF^{T1799A}, a retrovirus expressing mut BRAF shRNA (+). Cell lysates are separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to membrane, and probed with BRAF and tubulin antibodies. BRAF is not significantly affected in Mel1363 cells that have wt BRAF, but inhibited in 624Mel and WM35 cells that are heterozygous for mut BRAF (HET). Mut BRAF RNAi almost completely inhibited BRAF expression in A375 cells that are LOH of mut BRAF (Dong et al., 2003; Rotolo et al., 2005).

Other methods including real-time PCR, pyrosequencing, and allele-specific hybridization can be used to detect BRAF codon 600 mutations (e.g., reagents from Qiagen, Valencia, CA, USA; EntroGen, Tarzana, CA, USA; and Trimgen Sparks, Maryland, USA). Using various mutation assays, we and others reported high frequencies (approximately 70%) of BRAF mutations in benign melanocytic nevi (Dong et al., 2003; Pollock et al., 2003). This information is important in understanding BRAF mutation in tumor biology. Since nevi are much more common in the general population compared with the rare occurrence of melanoma, BRAF mutations alone are insufficient to cause malignant transformation in nevus cells. It has been shown that oncogenic BRAF cause proliferative senescence instead of unlimited proliferation in nevus cells (Michaloglou et al., 2005).

We designed mutant specific BRAF RNAi (siRNA and shRNA) to specifically target the T1799A mutant allele (Rotolo et al., 2005). Shown in Fig. 3 is the predicted mutant specific shRNA transcript, pRS-BRAF^{T1799A}, expressed from a retroviral vector pRETRO-SUPER-puro (pRS) (Brummelkamp et al., 2002). When a pRS-BRAF^{T1799A} construct is stably expressed in cultured melanoma cells using empty vector and a 19 bp firefly luciferase (luc) sequence as controls, BRAF expression is reduced in T1799A BRAF-positive but not in BRAF wild-type melanoma cells (Fig. 3B). Further, inhibition of BRAF expression is greatest in

A375 melanoma cells that show LOH at the BRAF locus and thus express only the T1799A mutant BRAF (Rotolo et al., 2005).

4. Loss of wild-type p16 and exogenous expression of INK4A in melanomas

Genetic and epigenetic lesions in the tumor suppressor gene INK4A are commonly found in cancer cells (Hanahan & Weinberg, 2000; Lowe & Sherr, 2003). Most melanomas, but not nevi, have lost the expression of wild-type INK4A either through DNA deletion/mutation or promoter hypermethylation (Castellano et al., 1997; Funk et al., 1998; von et al., 1999; Welch et al., 2001; Zhang et al., 2002). INK4A shares coding sequence with ARF in the CDKN2A locus (Fig. 4), but the proteins are translated in different reading frames (Sherr, 2001). ARF up-regulates p53 by interfering with the p53 negative regulator MDM2, while p16 binds to and inhibits cyclin-dependent kinases 4 and 6 (CDK4/6) promoting cell-cycle arrest via the RB tumor suppressor pathway (Chang et al., 2003; Lowe & Sherr, 2003). Although deletions and mutations may affect both INK4A and ARF genes, several studies have identified mutations in melanoma specimens affecting only INK4A without ARF involvement (Chin et al., 2006; Fargnoli et al., 1998). Consistent with a tumor suppressor role of INK4A in melanomas, a germ line Arg24Cys (R24C) mutation in CDK4 has been identified in familial melanoma patients (Wolfel et al., 1995; Zuo et al., 1996). This mutation abolishes CDK4 inhibition by p16 and thus is believed to be functionally equivalent to p16 loss. Cyclin D1 and D3 are over-expressed in melanomas, which is required for growth and survival of melanoma cells *in vitro* and *in vivo* (Bartkova et al., 1996; Florenes et al., 1996; Polsky & Cordon-Cardo, 2003). Mutational inactivation of RB tumor suppressor is rare in melanomas (Bartkova et al., 1996; Maelandsmo et al., 1996).

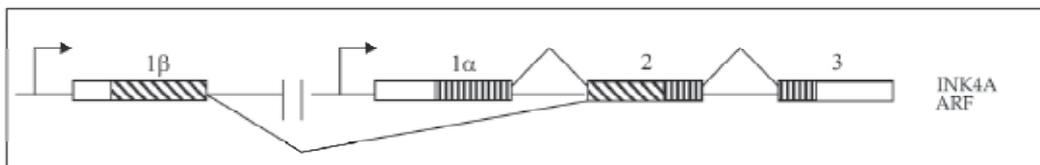


Fig. 4. Human CDKN2A locus. INK4A and ARF share sequences in the CDKN2A locus. The locus encodes two products, p16 and ARF (p14). Exons are shown as rectangles. Alternative first exons (1 α and 1 β) are transcribed from different promoters (arrows). The first exons are spliced to the same splicing acceptor site in exon 2 but are translated in alternative reading frames. INK4A coding sequences in exons 1 α , 2, and 3 and ARF coding sequences in exons 1 β and 2 are indicated by different shading patterns. Adopted from Sherr (Sherr, 2001).

We performed expression and mutation analyses of INK4A in melanoma cells. Immunohistochemical analysis shows that p16 is highly expressed in nevus cells (Fig. 5A), but expression decreases increasingly during tumor progression (Fig. 5B-D). Western blot analysis of cultured melanoma cells shows that p16 is barely detectable, if any, in melanoma cells (Fig. 5E). Mutation analysis of INK4A show that 624Mel cells have an 18 bp in-frame deletion of codons 32–37 (CTGGAGGCGGGGCGCTG) in exon 1 α . The deleted sequence is located in the first ankyrin repeat and encodes an evolutionarily conserved 6 amino acids (LEAGAL) (Greenblatt et al., 2003). Deletions and mutations affecting these amino acid have been reported in melanomas and other cancers and significantly affect the CDK- and cell cycle-inhibitory activities of p16 (Harland et al., 1997; Muzeau et al., 1997; Ruas & Peters,

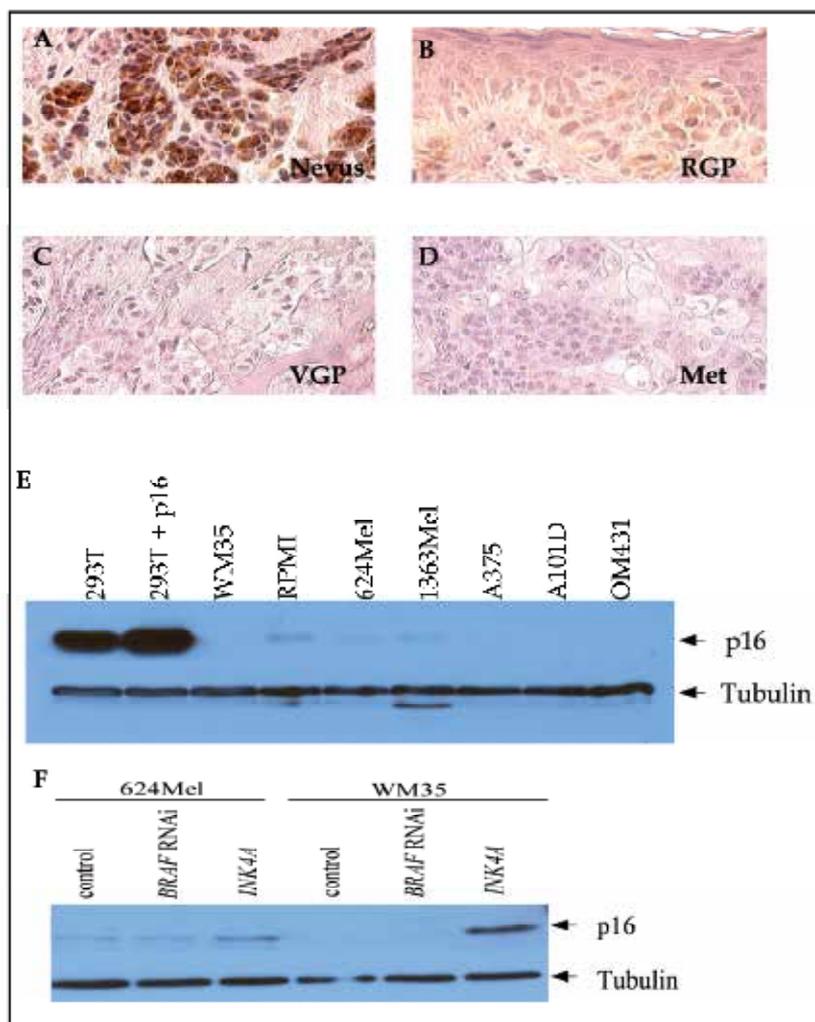


Fig. 5. Endogenous and exogenous expression of p16 in melanoma cells. (A-D) Immunohistochemical analysis of p16 expression in melanocytic lesions. Formalin-fixed and paraffin-embedded (FFPE) tissue sections of nevus (A), RGP (B), VGP (C), and metastatic (D) melanomas are stained with p16 antibody. All the samples are also positive for BRAF T1796A mutation (not shown). Note the strong expression of p16 in nevus but not in melanoma cells. Magnification $\times 100$. (E) Western blot analysis of p16 expression in melanoma cell lines. 293T control (293T), 293T transfected with INK4A cDNA (293T + p16) are used as controls. WM35, RPMI, 624Mel, 1363Mel, A375, A101D, and OM431 melanoma cell lines are cultured in regular media. Cell lysates are separated by SDS-PAGE. Western blot was probed with p16 and tubulin antibodies (arrows). WM35, RPMI, 624Mel, A375, A101D, and OM431 also have BRAF T1796A mutation. (F) Exogenous expression of wt INK4A in melanoma cells. Immunoblotting of 624Mel and WM35 controls (-), and cells stably infected with retroviruses expressing mut BRAF RNAi (BRAF RNAi) or INK4A cDNA (INK4A). Western blot is probed with p16 and tubulin antibodies (arrows). Note mutant BRAF inhibition has no detectable effect on endogenous (624Mel) or exogenous (WM35) p16 expression.

1998). The deletion shows LOH (data not shown), suggesting that either the wild-type copy of the gene is deleted or this is a homozygous deletion. Sequence analysis also shows that INK4A is wild-type in RPMI and 1363Mel and deleted in WM35 melanoma cells (data not shown). Mutant p16 is expressed at relatively lower levels in 624Mel cells compared to wild-type p16 in RPMI and 1363Mel cells (Fig. 5E). The expression of wild-type INK4A can be restored in melanoma cells by exogenous expression of INK4A cDNA (Rotolo et al., 2005; Zhao et al., 2008). As shown in Fig. 5F, 624Mel and WM35 melanoma cells are infected with either vector control or pBabe-neo-INK4A retroviruses. After G418 selection of mass culture, immunoblotting showed that p16 is expressed at approximately 3-5 fold more than endogenous p16 in 624Mel cells and at a higher level in WM35 cells (Fig. 5F).

5. Functional interaction of BRAF and INK4A lesions in melanoma cells

Activating BRAF mutations and loss of wild-type INK4A expression both occur at high frequencies in melanomas. However, BRAF and INK4A lesions can have overlapping roles in the regulation of the RB pathway (Fig. 6). As shown in Fig. 6, oncogenic BRAF can upregulate cyclin D through ERK pathway resulting in activation of CDK4/6. In contrast, p16 binds to and inactivates these CDKs. Therefore, CDK4/6 may be activated either by mutant BRAF through upregulation of cyclin D via ERK signaling or by loss of p16 activity. Activated CDKs phosphorylate and inactivate RB proteins resulting in the liberation of E2F transcription factors and cell cycle progression, which may contribute to the observed hyperphosphorylation of RB proteins and activation of E2F transcription in advanced melanoma cells (Halaban, 1999). There is also indirect evidence suggesting the cooperation between lesions of BRAF and INK4A in tumor development. In normal fibroblasts, oncogenic RAS and RAF have been shown to cause permanent growth arrest and/or senescence, rather than unrestricted proliferation, through a mechanism involving induction of INK4A expression (Lin et al., 1998; Zhu et al., 1998). Deficiencies in INK4A abrogate RAS-induced senescence, leading to transformation of human fibroblast cells (Brookes et al., 2002; Drayton & Peters, 2002; Drayton et al., 2003; Huot et al., 2002). In mice, neither oncogenic Ras nor Ink4a loss is sufficient to induce the development of melanomas. However, they generate spontaneous melanomas in combination (Chin et al., 1997).

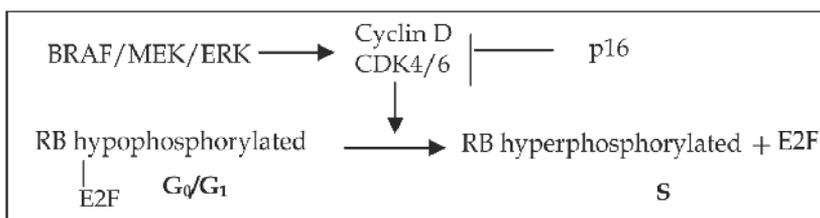


Fig. 6. The effects of BRAF and p16 can converge at the RB/E2F pathway through the opposite effects on cyclin-dependent kinases and cell cycle progression in the G₁/S phase.

6. Growth inhibition by either suppression of mutant BRAF or expression of wild-type INK4A

We performed experiments to examine the effects of inhibiting mut BRAF or expressing INK4A in melanoma cells. We found that 624Mel, A101D and WM35 cells that harbor both T1796A and INK4A mutations have intrinsic MEK activation (data not shown) that is

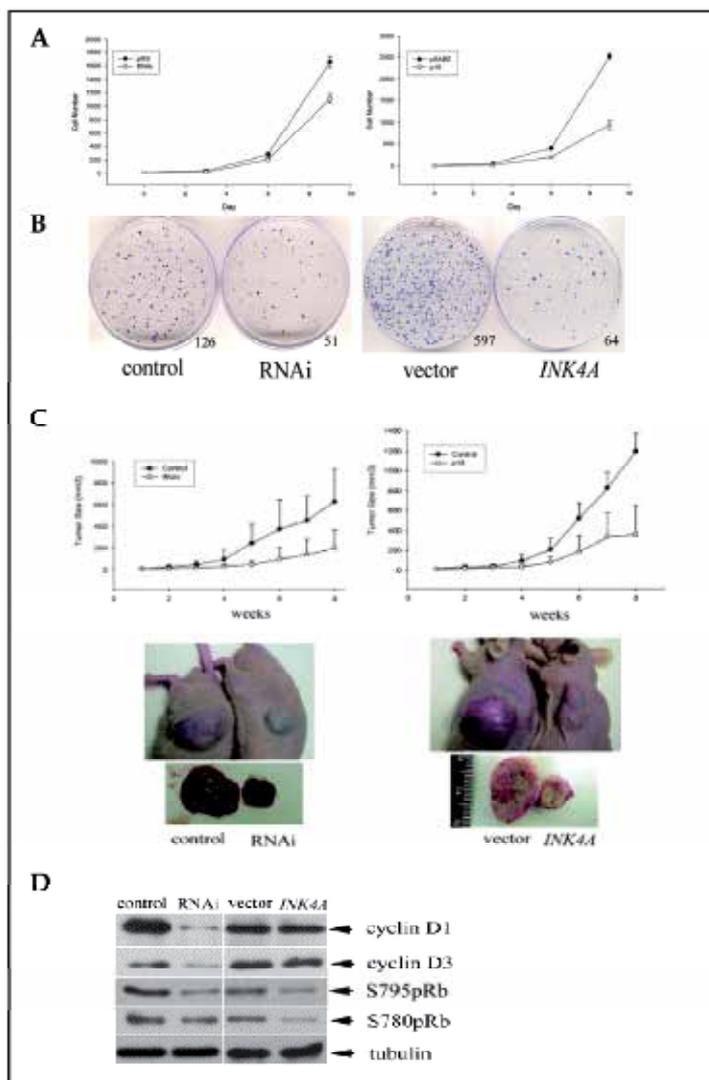


Fig. 7. Growth inhibition of melanoma cells by mutant BRAF RNAi or wild-type p16. Shown are *in vitro* and *in vivo* growth of 624Mel cells stably expressing either luc-RNAi (control) or mut BRAF RNAi (RNAi), vector control (vector) or wt INK4A (INK4A). (A) Cell counting. 5×10^4 624Mel cells are cultured and counted every 3 days using a hemocytometer ($P < 0.05$, Student *t* test of day 9 cells). (B) Colony formation assay. 1×10^3 624Mel cells are plated in triplicate in 35 mm diameter plates and grown for 2 weeks. Colonies are fixed and stained. The colony numbers shown are the average colony counts from three plates ($P < 0.001$, Two Poisson Parameters test). (C) Mouse xenograft assay. 5×10^5 624Mel cells are injected subcutaneously into flanks of nude mice ($n = 6$). Tumor growth is monitored. Pictures are taken 8 weeks after cell inoculation. The average tumor volumes are calculated and plotted ($p < 0.01$, *t* test). (D) Expression of cyclin D1/D3 and phospho-pRB. Western blotting is performed using cell lysates from 624Mel control cells and cells expressing mut BRAF RNAi or INK4A and probed with cyclin D1 and cyclin D3 or Ser795 and Ser780 phosphorylated pRB (Rotolo et al., 2005).

inhibited by mut BRAF RNAi (Rotolo et al., 2005), consistent with earlier reports (Hingorani et al., 2003; Karasarides et al., 2004; Sumimoto et al., 2004; Wellbrock et al., 2004). Both mutant BRAF RNAi and wild-type INK4A significantly inhibited the growth of 624Mel cells in tissue culture, as measured by cell count (Fig. 7A) and colony formation assay (Fig. 7B). Population doubling times of mut BRAF RNAi and wild-type INK4A expressing 624Mel cells are on average 36 hr and 50 hr, respectively, compared to controls of approximately 24 hr (Rotolo et al., 2005). The effect on tumorigenesis is examined in a nude mice xenograft assay. Tumor growth is significantly inhibited by both mut BRAF shRNA and wild-type INK4A in 624Mel (Fig. 7C) and A101D and WM35 cells (not shown). Consistent with the observed growth inhibitory effects and with an overlapping role of BRAF and INK4A lesions in RB protein and cell cycle regulation (Fig. 6), cyclin D1 and D3 are downregulated in cells expressing mut BRAF RNAi, and phosphorylation of S780 and S795 of pRB, known cyclin D1/CDK4 targets are suppressed by both BRAF RNAi and INK4A (Fig. 7D).

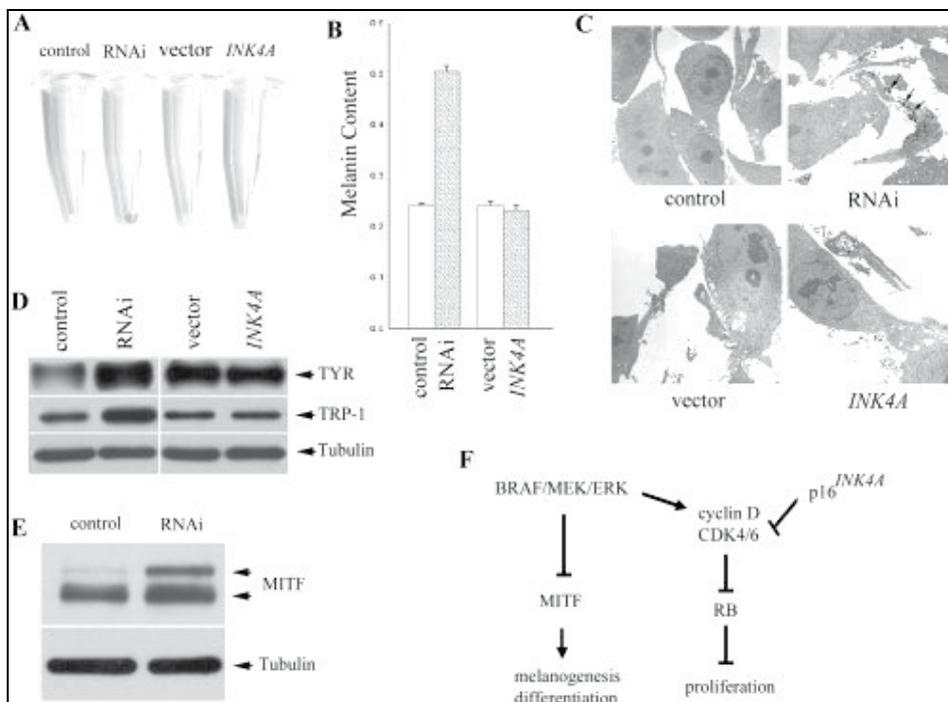


Fig. 8. Effect on melanogenesis. (A) Color of cell pellets. 5×10^6 624Mel controls and cells expressing mut BRAF RNAi or INK4A are pelleted. RNAi pellet shows a visible darker color. (B) Melanin contents; 5×10^6 cells are measured for melanin content. Data are means \pm SE from 3 experiments performed in triplicate ($p < 0.001$, t test). (C) Representative electron micrographs of cultured 624Mel controls and cells expressing mut BRAF RNAi or INK4A. Note the increased numbers of mature melanosomes in RNAi-expressing cells (arrows). Magnification $\times 2,000$. Similar changes are obtained using dissected xenograft tumors. (D) Western blot analysis of the expression of tyrosinase (TYR) and TRP-1 proteins. Western blot is probed with TYR, TRP-1 and α -tubulin antibodies. (E) Increased MITF in cells expressing T1796A BRAF RNAi, shown by Western blotting. (F) Model of separate regulation of proliferation and differentiation by mutant BRAF and p16 in melanoma cells.

Melanogenesis, a sign of melanocyte differentiation, is often changed in melanomas (Halaban, 2002). During normal development, differentiation stimuli trigger the activation of microphthalmia-associated transcription factor (MITF) to promote cell-cycle arrest and initiate the melanogenesis process (Fang & Setaluri, 1999; Setaluri, 2003; Widlund & Fisher, 2003). Melanogenesis is a multi-step biochemical process resulting in the formation of melanin in pigment cells. Melanogenic factors, tyrosinase (TYR), and tyrosinase-related protein-1 (TRP1) participate in the melanogenic pathway and are important melanocyte differentiation markers (Fang & Setaluri, 1999; Setaluri, 2003). Eberle et al (Eberle et al., 1995) compared the expression of TYR and TRP1 in cultured normal human melanocytes and melanoma cell lines by Northern blotting and reverse transcriptase-PCR (RT-PCR). They found that TYR and TRP1 genes are expressed in normal human melanocytes, but the expression is repressed in nearly all of the 14 melanoma cell lines examined and is completely absent in 4 of the 14 lines (Eberle et al., 1995). Hofbauer et al (Hofbauer et al., 1998) also found that tyrosinase expression level correlates inversely with clinical stage. These data suggest that the normal melanogenesis program is inhibited in melanoma cells. It is believed that differentiation and malignancy are inversely correlated and cancer is a disease of cell differentiation (Harris, 2004). Although unlimited proliferation and defects in cellular differentiation are characteristic of cancer growth, how the abnormal proliferation and pigmentation/differentiation are interconnected in the development of melanoma and other cancers is largely unknown (Coffman & Studzinski, 1999; Halaban, 2000).

Suppression of mutant BRAF inhibited ERK signaling, which may be attributed to the observed melanogenic effects. However, both activation and inhibition of ERK signaling can cause increased pigmentation. For example, although melanogenesis in melanocytes is suppressed both *in vitro* and *in vivo* by exogenous expression of Ras oncogene (Dotto et al., 1989; Tsukamoto et al., 1992; Wilson et al., 1989), transgenic mice expressing Ras under a mouse tyrosinase promoter demonstrated hyperpigmentation and melanocytic hyperplasia (Powell et al., 1995). Activation of ERK signaling by the c-Kit receptor plays a crucial role in the differentiation of pigment cells during development (Lassam & Bickford, 1992), whereas constitutive ERK signaling in melanoma cells is inhibitory to melanogenesis and inhibition of intrinsic ERK signaling in melanoma cells causes induction of melanogenesis and cellular differentiation (Englaro et al., 1998; Kim et al., 2004). Although our results demonstrate a melanogenic effect by inhibiting mutant BRAF, it is worth noting that activating BRAF mutations have been found in 70–80% of benign nevi that are mostly dark in color, and some malignant melanomas that are highly pigmented may have BRAF mutation. Thus, although BRAF mutation is important in blocking the melanogenic process in melanoma cells, it is not sufficient by itself to cause depigmentation. It is clear that the roles of BRAF mutation and ERK signaling in melanogenesis and melanocytic differentiation depend on the cellular context. Precisely what role is played by BRAF mutation and how it interacts with other signaling pathways in the determination of pigmentation phenotype needs to be further studied.

Since restoring p16 expression does not increase pigmentation, though cell proliferation is also suppressed, the differentiation effect of BRAF inhibition is not merely the result of general growth suppression. Rather, mutant BRAF actively participates in the differentiation program while simultaneously inducing proliferation. It is well known that malignant cells, including those of melanomas, maintain their differentiation program and sensitivity to differentiation modulators (Leszczyniecka et al., 2001). Therefore, understanding mutant BRAF in the regulation of the differentiation process may provide strategies by targeting

mutant BRAF to reverse melanoma cells to a more mature and less malignant state. Melanogenesis is induced by inhibition of mutant BRAF but not by expression of wild-type p16, suggesting the existence of different mechanisms and nonoverlapping roles of the two most common genetic lesions in the malignant transformation of melanoma.

7. Growth inhibition and apoptosis by simultaneous inhibition of mutant BRAF and expression of INK4A in melanoma cells

Aberrant BRAF and INK4A often co-exist in melanoma and the regulatory relationship between BRAF and INK4A is unclear. It is possible that the co-occurrence is a result of genetic changes necessary for melanoma development without functional significance in melanoma cells. For example, in benign nevi, mutant BRAF turns on the expression of p16 resulting in proliferative senescence to counteract BRAF's proliferative drive (Bennett, 2008). Thus, loss of wild-type p16 is permissive to BRAF initiated melanoma development (Bennett, 2008). If mutant BRAF is still capable of turning on INK4A in melanoma (as in benign nevi), suppression of mutant BRAF, which has been actively explored to treat melanoma, may actually reduce the expression of any remaining functional p16 in melanoma cells and counteract the inhibitory effect of the treatment. On the other hand, the non-overlapping roles in melanogenesis suggest non-epistatic and additive/synergistic activities by the co-existing lesions. We hypothesize that simultaneous inhibition of BRAF-MEK and INK4A-CDK4 pathways is more effective than either alone in melanoma treatment. To test this hypothesis we performed experiments to co-express mut BRAF RNAi and INK4A in melanoma cells. Our initial studies revealed a sequence dependent difference when mut BRAF shRNA and wt INK4A are stably expressed in melanoma cells; the orders of introducing mut BRAF shRNA and wt INK4A generate different outcomes (Table 1, Table 2). 624Mel and WM35 cells stably expressing mut BRAF RNAi followed by wt INK4A expression (Set 1 in Table 1) are lethal although control 1363Mel cells are viable (Set 1 in Table 2). Whereas 624Mel and WM35 viable lines are eventually generated after weeks of tissue culture in experiments performed in the reverse order (Set 2 in Table 1 and Table 2). These results indicate that suppression of mut BRAF followed by restoration of wt INK4A generates more inhibitory effect than experiment performed in the reverse order in cells that harbor BRAF and INK4A lesions. We propose that inhibition of mut BRAF induces melanoma cells to a more differentiated state more susceptible to suppression by INK4A, whereas prior expression of INK4A interferes with differentiation program by mut BRAF inhibition. Therefore, the order of interventions could be important when targeting both BRAF and INK4A lesions in melanoma treatment.

Set 1*	Established line	pRS-puro	pRS-puro BRAF shRNA
	2 nd control	pBabe-neo	pBabe-neo
	2 nd Infection	pBabe-neo-INK4A	pBabe-neo-INK4A
Set 2*	Established line	pBabe-neo	pBabe-neo-INK4A
	2 nd control	pRS-puro	pRS-puro
	2 nd Infection	pRS-puro BRAF shRNA	pRS-puro BRAF shRNA

Table 1. Generation of melanoma cell lines expressing both mutant BRAF RNAi and INK4A

Cell line*	Set 1*		Set 2*	
624Mel	control	viable	control	viable
	BRAF shRNA + INK4A	lethal	INK4A + BRAF shRNA	viable
WM35	control	viable	control	viable
	BRAF shRNA + INK4A	lethal	INK4A + BRAF shRNA	viable
1363Mel	control	viable	control	viable
	BRAF shRNA + INK4A	viable	INK4A + BRAF shRNA	viable

*Set 1 and Set 2 experiments are performed in the reverse order. (Set 1) Cells infected and selected to stably expressing mut BRAF shRNA (pRS-puro BRAF RNAi) are subsequently infected with retrovirus pBabe-neo-INK4A. Cells are then under puro- and G418 double selection. (Set 2) Cells infected and selected to stably expressing pBabe-neo-INK4A are subsequently infected with retrovirus pRS-puro BRAF shRNA, and then double selected with puro- and G418. 624Mel and WM35 cells are positive for both BRAF and INK4A mutations, whereas 1363Mel is wt for both BRAF and INK4A.

Table 2. The sequence of expression of mut BRAF RNAi and wt INK4A affects cell survival

Melanoma cells under lengthy puro and G418 double selection seem to undergo various changes (e.g., change in ploidy as measured by flow cytometry, data not shown). We therefore choose to examine the more direct effect using transient transfection experiment. As described in Fig. 2 and Fig. 5, human melanoma cell line 624Mel is heterozygous for BRAF T1799A mutation (the ratio of T1799:A1799 alleles is about 1:1) and the cells have a six amino acid deletion in the exon 2 of INK4A although the smaller mutant protein is still detectable by Western blotting. Expression of BRAF RNAi and p16 cDNA each individually caused comparable levels of growth inhibition in melanoma cells through down-regulation of RB phosphorylation at the CDK4/6 sites (Fig. 7). 624Mel cells are transiently transfected with scrambled siRNA (5'--AAG UCC AUG GUG ACA GGA GAC-3') and pBabe vector (control), BRAF siRNA (5'--AAG UGG CAU GGU GAU GUG GCA-3') and pBabe vector (siRNA), scrambled siRNA and INK4A cDNA (INK4A), and BRAF siRNA and INK4A cDNA (siRNA-INK4A). The transfection efficiencies in 624Mel cells are approximately 30-50%, and the effects of transfection are assessed on unselected mass cultures. The RAS-RAF-MEK-ERK signaling pathway is viewed as upstream of the cyclin D-CDK4/6-p16-RB cascade; consistently, only melanomas with wild-type NRAS/BRAF have amplified CDK4 and cyclin D1 genes (Curtin et al., 2005). Therefore, expression of p16 may be epistatic to down-regulation of BRAF. If so, we would expect similar effects in cells expressing BRAF siRNA, INK4A cDNA, or BRAF siRNA plus INK4A cDNA. However, we found that simultaneous expression of BRAF siRNA and wild-type INK4A (siRNA-INK4A cells) inhibit cell growth more than either alone (siRNA or INK4A cells) as measured by cell counting (Fig. 9A, $p < 0.0001$, ANOVA; Tukey's Studentized range (HSD) test at 0.05 significance level) (Zhao et al., 2008) and colony formation (Fig. 9B).

Expression of BRAF siRNA and INK4A cDNA caused corresponding changes on levels of BRAF and p16 as detected by immunoblotting (Fig. 9D). BRAF levels are reduced in cells expressing BRAF siRNA (siRNA) and BRAF siRNA plus INK4A cDNA (siRNA-INK4A) but not in cells expressing INK4A cDNA (INK4A). The expression of p16 is increased in INK4A-transfected cells, and not in cells transfected with BRAF siRNA alone (Fig. 9D). Expression of BRAF siRNA caused reduction of MAPK signaling as indicated by ERK phosphorylation relative to ERK abundance (Fig. 9D), suggesting that the effects of BRAF inhibition are mediated by the BRAF-MEK-ERK signaling cascade. Since CDK4/6 kinases can be activated not only by RAF-MEK-ERK signaling, but also by other signaling pathways such

as PI3K-AKT (Meier et al., 2007), exogenous p16 may generate broader inhibition of CDK4/6 activity not limited to that regulated by RAF-MEK-ERK. BRAF siRNA, on the other hand, may aid in p16 growth inhibition through reduced baseline activity of cyclin D-CDK4/6. Additionally, we found that levels of p27KIP1 are increased in siRNA, INK4A, and siRNA-INK4A cells (Fig. 9D). The changes of p27KIP1 are in line with the observed growth inhibition and with the well-established role of p27KIP1 in cell cycle regulation. p27KIP1, a negative regulator of cyclin E-CDK2, together with the reduced CDK4/6 activity as a result of BRAF siRNA and p16 expression, may block RB phosphorylation at both the CDK4/6 and CDK2 sites (note CDK2 level is not significantly altered, Fig. 9D), allowing RB to form complex with E2F and block cell cycle progression. p27KIP1 is regulated through different mechanisms (Bhatt et al., 2005), and further studies are required to understand the molecular mechanisms of the growth inhibition by BRAF siRNA and INK4A.

Induction of apoptosis by BRAF siRNA has been observed in some melanoma cells (Hingorani et al., 2003; Sumimoto et al., 2004), whereas p16 may act to block apoptosis (Maddika et al., 2007). To test whether expression of INK4A cDNA interferes with apoptosis induction by BRAF siRNA, we performed a TUNEL assay 72 h after transfection of 624Mel cells with BRAF siRNA and/or INK4A cDNA. Neither inhibition of BRAF siRNA nor expression of INK4A cDNA lead to significantly higher apoptosis than control (Fig. 10C, Tukey's Studentized range (HSD) test at 0.05 significance level does not detect statistically significant difference of apoptosis between siRNA, INK4A and control). However, coexpression of BRAF siRNA and INK4A cDNA triggered statistically significant difference of apoptosis (Fig. 9C, siRNA-INK4A in 624Mel cells generated significantly higher apoptosis than control or siRNA or INK4A, ANOVA $p = 0.0003$ and $p < 0.0001$, respectively; Tukey's Studentized range (HSD) test at 0.05 significance level). Note that there is no selection for transfected cells, so the maximum expected apoptosis is 50% (equivalent to the maximum transfection efficiency). The apoptotic effect, together with the enhanced growth inhibition (Fig. 9A and 9B), are consistent with the difficulty in generating stable cell lines expressing both BRAF short hairpin RNA and INK4A cDNA (Table 1 and Table 2). An anti-apoptotic effect of p16 has been previously reported that may be functionally equivalent to activation of RB since functional RB is known to suppress apoptosis (Harbour & Dean, 2000; Pucci et al., 2000). However, RB phosphorylation should be further inhibited in siRNA-INK4A expressing cells. The observed apoptosis may be mediated by other molecule(s) that over-ride or modify the anti-apoptotic effect of RB. Proteins in the BCL2 family are known to be critical regulators of apoptosis (Adams & Cory, 2007) which is in line with our observation that apoptosis is associated with the pro-apoptotic protein BIM (BCL2 interacting mediator of cell death (Collins et al., 2005), and BCL2, a critical prosurvival factor (Fig. 9D). The pro- and antiapoptotic counterparts of the BCL2 protein family can form heterodimers and neutralize each other's functions, suggesting that their relative concentrations play a pivotal role in the execution of programmed cell death. In BRAF siRNA cells, only an increase in BIM is detected, whereas in siRNA-INK4A cells, increased BIM occurs together with a decreased level of BCL2 (Fig. 9D). This could further offset the balance toward activation of apoptosis. The decrease in the levels of BCL2 protein is observed only in siRNA-INK4A cells but not in siRNA or INK4A cells suggesting a functional interaction between BRAF and p16 in the regulation of this pro-survival protein. Given the critical role of p53 in apoptosis, we performed sequencing analysis of p53 cDNA and found that 624Mel cells harbor a T1076G (Cys275Trp) mutation in the DNA binding domain that is likely to compromise the transcription and apoptosis function of p53 (Petitjean et al., 2007), suggesting that the observed apoptosis may not necessarily involve p53.

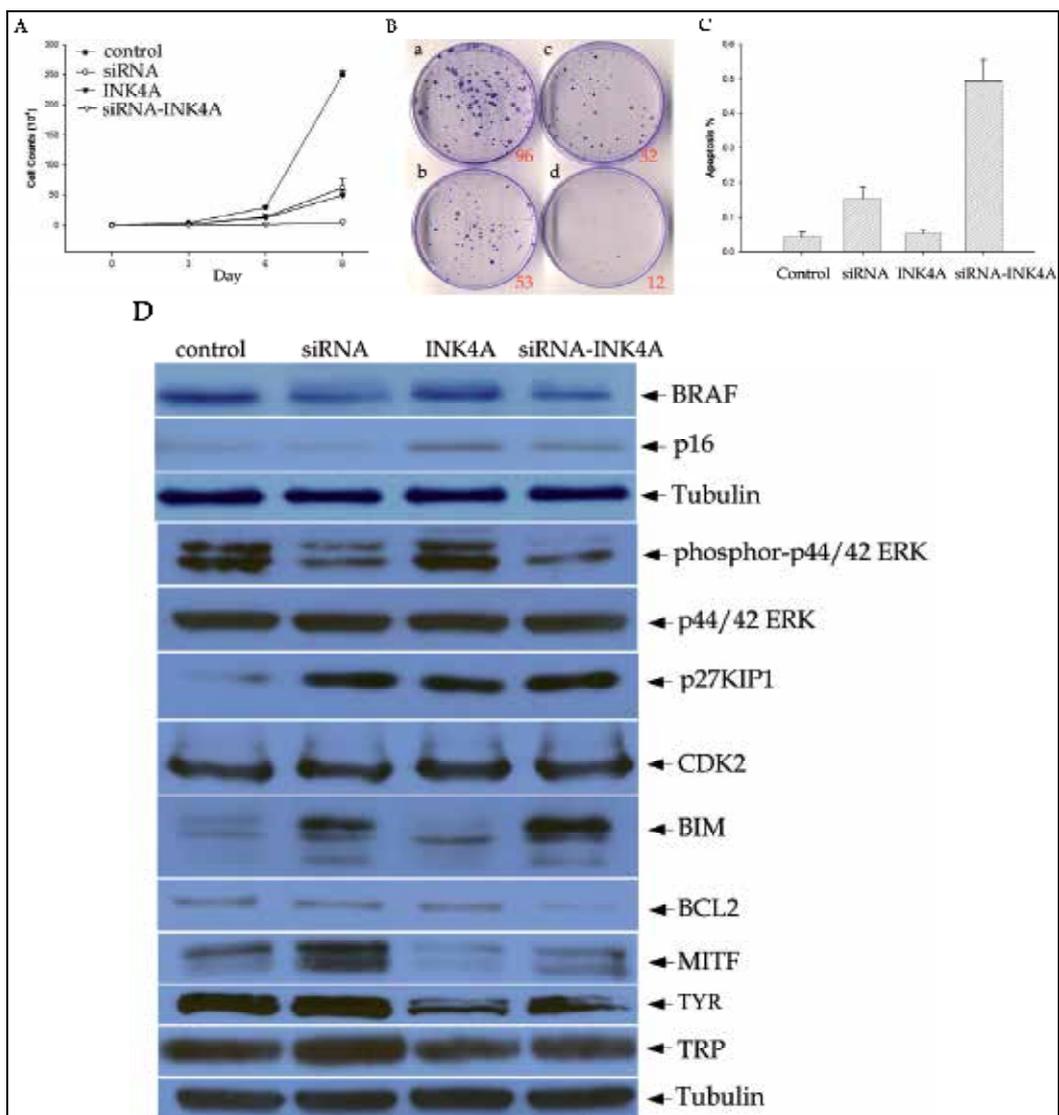


Fig. 9. Effects of simultaneous inhibition of BRAF by siRNA and expression of INK4A cDNA in 624Mel melanoma cells. 624Mel melanoma cells are transiently transfected with scrambled siRNA and pBabe vector (control), BRAF siRNA and pBabe vector (siRNA), scrambled siRNA and INK4A cDNA (INK4A), and BRAF siRNA and INK4A cDNA (siRNA-INK4A). (A) Growth inhibition measured by cell counting at day 3, 6, and 9 after transfection. (B) Growth inhibition measured by colony formation 2 weeks after transfection. (C) Apoptosis detected by TUNEL assay 72 h after transfection. (D) The expression of BRAF, p16 and several other proteins that are key regulators of proliferation (phosphor-p44/42 ERK, p27KIP1, CDK2), apoptosis (BIM, BCL2), and melanocytic differentiation (MITF, TYR, TRP1) are examined by Western blotting 48 hr after transfection. Tubulin is used as loading control (Zhao et al., 2008).

The mechanisms involved in the observed combinatory inhibition of proliferation and survival needs to be further explored. As the induced apoptosis is concomitant with growth arrest, it is possible that they are related. Alternatively, different cell populations, such as cells in different phases of the cell cycle, may respond differentially to BRAF siRNA and p16 under the same experimental condition. The observed growth inhibition and apoptosis may not simply be a quantitative/additive effect on CDK-RB since BRAF-MEK-ERK signaling has multiple targets that are not limited to CDK-RB regulation. Additionally, BRAF inhibition and p16 expression have different effects on melanogenesis (Fig. 8), suggesting that p16 may have qualitatively different activities not necessarily overlapping with BRAF, which may be the basis for the observed combinatory effects. In summary, our results show that simultaneous inhibition of mutant BRAF and expression of wild-type p16 cooperates in the inhibition of proliferation and enhances apoptosis, suggesting that BRAF and INK4A lesions - two of the most common genetic abnormalities in melanomas, interact functionally in melanoma cells and that strategies designed to correct both lesions could be effective for melanoma treatment.

8. Inhibition of MEK and CDK4 in melanoma cells

It has been shown that BRAF and INK4A may have activities independent of the corresponding canonical ERK and RB pathways, and the two pathways also mediate cellular signals independent of aberrant BRAF and INK4A. For example, RAF can act through apoptosis signal-regulating kinase-1 (ASK1)/c-Jun-NH2-kinase or mammalian sterile 20-like-kinase 2 (MST2) pathways (O'Neill et al., 2005); cyclin D:CDK4/6 can be activated by enhanced phosphatidylinositol 3-kinase (PI3K) and WNT signaling pathways in melanomas (Delmas et al., 2007; Schmitt et al., 2002). To examine whether the combinatorial effects of BRAF RNAi and INK4A cDNA in melanoma cells is specific to BRAF and INK4A or can be generalized to other components of the ERK and RB pathways, we tested PD98059 and 219476, commercially available inhibitors of MEK and CDK4, respectively, in human melanoma cells. Of note, deregulation of the RAS-RAF-MEK-ERK (ERK) and p16-cyclin D:CDK4/6-RB (RB) pathways are common in human malignancies and appears to be important for melanoma development. As shown in Fig. 1, chemotherapeutic agents targeting components of both pathways have been developed but clinical studies with monotherapy have been disappointing.

Human melanoma cell lines 624Mel, A101D, and A375 harbor heterozygous BRAF T1799A mutation and loss of wild-type p16 (Rotolo et al., 2005). The cells are treated, alone or in combination, with MEK inhibitor PD98059 (Waters et al., 1995) and CDK4 inhibitor 219476 (Zhu et al., 2003). As anticipated, ERK phosphorylation is reduced in cells treated with PD98059 and PD98059 plus 219476 (Fig. 10A). Levels of p27KIP1, a negative regulator of cyclin E:CDK2, are increased in cells treated with either PD98059 or 219476, and further increased in cells with combinatorial treatment (Fig. 10B). Phosphorylation of S780, S795, and S807/811 of RB, known cyclin D:CDK4 and cyclin E:CDK2 targets (Halaban, 2005), is decreased in cells treated with either PD98059 or 219476 (except S780 and S807/811 in OM431 cells), and further reduced in cells with combinatorial treatment (Fig. 10C). Of note, total RB is decreased under combinatorial treatment with PD98059 and 219476 in all three melanoma cells (Fig. 10C).

PD98059 and 219476 inhibit tumor cell growth in a dose dependent manner (Krasilnikov et al., 2003; Zhu et al., 2003). In order to make it possible to monitor the additional therapeutic

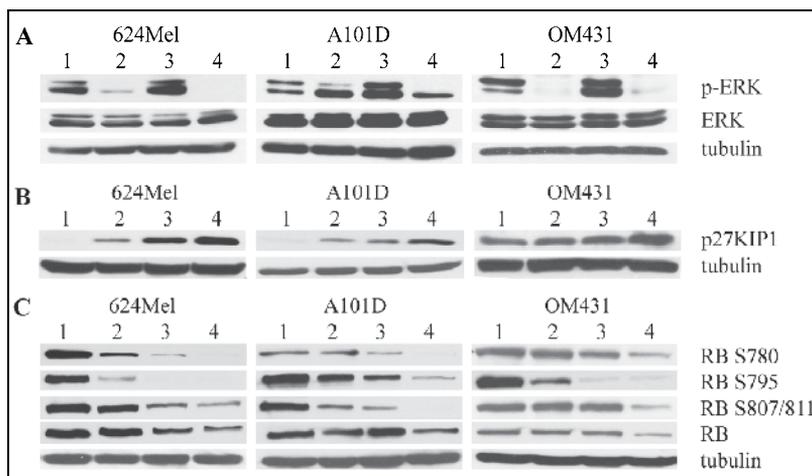
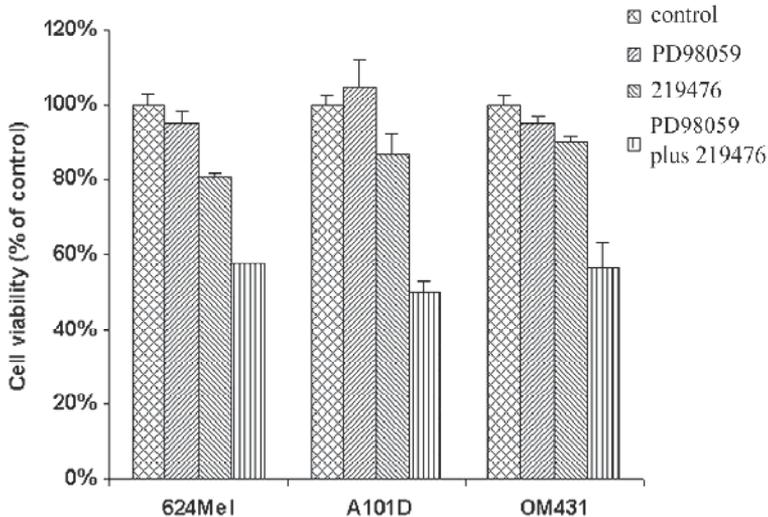


Fig. 10. Regulation of ERK phosphorylation, p27KIP1 expression, and RB phosphorylation by PD98059 and 219476, alone and in combination. Human melanoma cell lines 624Mel, A101D, and OM431 are treated with either vehicle solvent (1), PD98059 (2), 219476 (3), or PD98059 plus 219476 (4) (Li et al., 2010a). Western blot is performed using 20 μ g total cell lysates. Tubulin is used as the loading control.

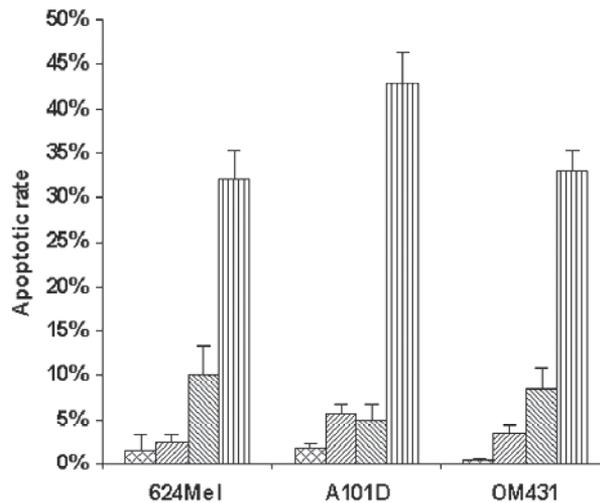
effects of the combinatorial treatment, both chemicals are used at dosages lower than that which would lead to maximal effect by either agent. The cytotoxicity of PD98059 and 219476 is measured using the MTS assay that measures the dehydrogenase enzyme activity found in metabolically active cells. In all the three cell lines, a significant difference in MTS counts exists for the control, PD98059, 219476, and PD98059 plus 219476 groups ($p < 0.0001$, R-Square 0.981444, 0.956956, and 0.991102 in 624Mel, A101D, and OM431, respectively, ANOVA). Further analysis shows that simultaneous treatment with PD98059 and 219476 results in significantly reduced numbers of cell survival than control or mono-treatment as measured by MTS in all the three cell lines (Fig. 11A, Tukey's Studentized Range (HSD) Test at 0.05 significance level). TUNEL DNA fragmentation assay is used to identify loss of viability due to programmed cell death. As shown in Fig. 11B, at the drug concentrations used, significantly different levels of apoptosis exist among control for PD98059, 219476, and combinatorial treatment groups ($p < 0.0001$, R-Square 0.973862, 0.990697, and 0.987900 in 624Mel, A101D, and OM431, respectively, ANOVA). Treatment with PD98059 alone results in no difference in apoptosis over controls in all three cell lines; 219476 enhances apoptosis in OM431 but not in the other two cell lines. However, combined treatment dramatically increases apoptosis over that seen for the control and mono-treatments (Fig. 11B, Tukey's Studentized Range (HSD) Test at 0.05 significance level).

We examined the expression of several pro- and anti-apoptotic proteins. Mono-treatment with PD98059 or 219476 causes a decreased or no change in the expression of anti-apoptotic proteins BCL2, BCL2L1, and BIRC5. While there are variations in the patterns of expression of BCL2, BCL2L1, and BIRC5 among the different cell lines, combinatorial treatment causes a comprehensive down-regulation of the proteins in all three cell lines (Fig. 12). In addition apoptosis facilitator BIM-EL is increased following treatment with PD98059 and PD98059 plus 219476 in all three cell lines. It is also increased in OM431 cells following treatment with 219476 (Fig. 12). Consistent with increased apoptosis, caspase 3 is activated by simultaneous

treatment with PD98059 plus 219476 in all three cell lines, as shown by decreased pro-caspase 3, increased levels of the active form of caspase 3 (cleaved caspase 3), and degradation of PARP, a direct substrate of active caspase 3 (Fig. 12).



(A)



(B)

Fig. 11. Cytotoxicity by MEK inhibitor PD98059, CDK4 inhibitor 219476, and combinatorial treatment. (A) MTS cytotoxicity assay is performed in 624Mel, A101D and OM431 cells after 48h treatment in medium supplemented with 0.5% FBS. The results are given as means \pm SD from three independent tests. (B) MEK and CDK4 inhibitors induce apoptosis of melanoma cells. TUNEL assay is performed in 624Mel, A101D and OM431 cells after 48h treatment with vehicle solvent, PD98059, 219476, or PD98059 plus 219476 in medium with 0.5% FBS. The results are given as means \pm SD from three independent assays.

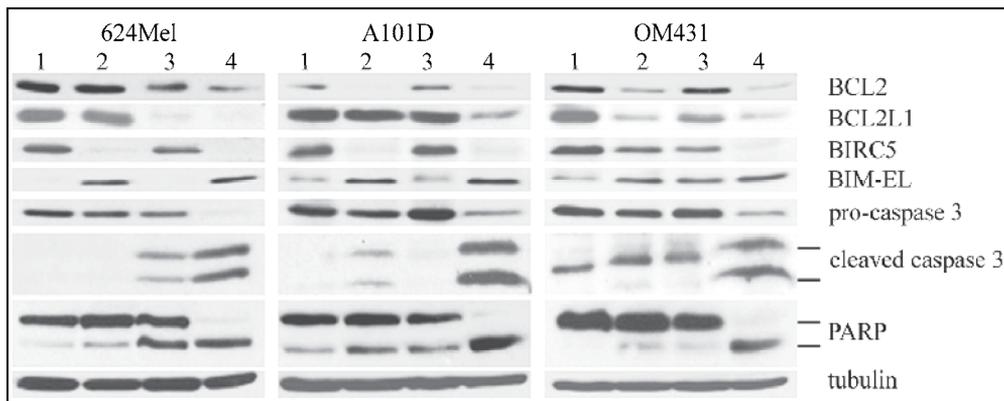


Fig. 12. Western blot analysis of changes in the expression of pro-survival and pro-apoptotic proteins. Cells are treated with solvent vehicle control (1), PD98059 (2), 219476 (3), and PD98059 plus 219476 (4) for 48 h in medium containing 5% FBS. 20 μ g total cell extracts from 624Mel, A101D and OM431 cells are separated by SDS-PAGE and blotted using BCL2, BCL2L1, BIRC5, BIM, caspase-3, and PARP antibodies. Tubulin is used as loading control.

It has been well-established that constitutive activation of the ERK signaling induces the expression of cyclin D (Michaloglou et al., 2008; Rotolo et al., 2005), which binds to and activates CDK4 leading to the phosphorylation of RB protein facilitating cell cycle entry (Halaban, 2005). Consistent with an epistatic regulation between ERK pathway and cyclin D:CDK4, amplification of cyclin D1 and CDK4 genes have been identified mainly in melanomas that harbor wild-type NRAS and BRAF (Curtin et al., 2005; Smalley et al., 2008). Additionally, cyclin D:CDK4 can mediate resistance to inhibitors of the ERK signaling pathway (Smalley et al., 2008). Therefore, the enhanced apoptosis and decreased proliferation by simultaneously inhibiting ERK and RB pathways could result from the double hitting of ERK-cyclin D:CDK4-RB that regulate cell cycle progression and cell survival. Alternatively, in support of our previous results that BRAF and INK4A have a non-linear functional interaction (Rotolo et al., 2005; Zhao et al., 2008), additional cellular processes could be affected when cells are exposed to both PD98059 and 219476. ERK pathway has pleiotropic activities that regulate cell proliferation, survival, and differentiation through both cyclin D:CDK4 dependent and independent routes (Fecher et al., 2008; Panka et al., 2006a; Sekulic et al., 2008). Likewise, cyclin D:CDK4 can be regulated and converges multiple cellular signals. For example, while PI3K signaling can activate CDK4 through down-regulation of INK4A and up-regulation of cyclin D (Schmidt et al., 2002), WNT signaling can turn on CDK4 through suppression of INK4A transcription (Delmas et al., 2007). It is conceivable that inhibition of MEK and CDK4 not only affects ERK and RB pathways, but also PI3K, WNT, and other ERK signaling activities not mediated through the RB pathway. Therefore, simultaneous targeting of both ERK and RB pathways can generate enhanced effects by targeting both linear and non-overlapping activities.

Our results show that simultaneous inhibition of MEK and CDK4 using pharmacological inhibitors PD98059 and 219476 leads to significantly increased apoptosis compared to control and single agent treatment (Figs. 11, 12). This effect is consistent with results observed after simultaneous knockdown of mutant BRAF using siRNA and expression of INK4A cDNA in melanoma cells (Fig. 9). The apoptotic effect is associated with changes of

apoptosis related proteins (Figs. 9 and 12). BCL2L1 and BIRC5 are highly expressed in melanoma cells, and increased expression correlates with tumor progression (Piras et al., 2007; Zhuang et al., 2007). A straightforward explanation for the observed apoptosis is that changes in the pro- and anti-apoptotic factors offset the balance and lead to apoptosis (Adams & Cory, 2007). Sequencing analysis of TP53 cDNA (Zhao et al., 2008) showed that 624Mel and OM431 cells harbor a T1076G (Cys275Trp) and a G1048A (Gly266Glu) mutations, respectively, in the DNA binding domain that is likely to compromise the transcriptional and apoptotic function of p53 (Petitjean et al., 2007). No TP53 mutation has been detected in A101D cells. Although apoptosis is enhanced in all three cell lines, it is somewhat more pronounced in A101D than 624Mel and OM431 cells treated with PD98059 and 219476 (Fig. 11), suggesting that TP53 status may influence the magnitude of apoptosis. Combinatorial treated cells have further inhibited phosphorylation of ERK and RB, reduced total RB, and increased expression of p27KIP1 (Figs. 9 and 12). Yu et al. demonstrated that loss of Rb causes apoptosis without effecting cellular proliferation (Yu et al., 2003) and Wang et al. found that over-expression of p27KIP1 leads to apoptosis in melanoma cells (Wang et al., 1997). The mechanisms of these changes in relationship to each other and to the observed cooperative effects need to be further investigated.

Apoptosis resistance is a critical factor for therapy failure in melanoma patients. Encouragingly, our results demonstrate that an increase in apoptosis can be achieved through combinatorial targeting of BRAF-MEK-ERK and p16-CDK4-RB pathways. Deregulation of the RAS-RAF-MEK-ERK and p16-cyclin D:CDK4/6-RB pathways are common in human malignancies and appears to be important for melanoma development. There has been significant effort to target components of these pathways in cancer treatment. Pharmacologic agents targeting components of the ERK and RB pathways have been developed. However, clinical studies using monotherapy show that the clinical responses have failed expectations and maximum tolerated doses are often reached before reaching clinical efficacy (Burdette-Radoux et al., 2004; Fecher et al., 2008; Panka et al., 2006b; Sekulic et al., 2008). Our study suggests that combination targeting of ERK and RB pathways is a promising strategy for melanoma treatment and should encourage further in-depth investigations.

9. HERV-K expression correlates with MEK and CDK4 activation

The K type human endogenous retroviral sequence (HERV-K) is expressed in melanoma cells but not in melanocytes (Muster et al., 2003). Through millions of years of evolution and natural selection, HERVs have become indispensable parts of the human genome. For example, syncytin-1 and syncytin-2, encoded by the envelope (ENV) genes of HERV-W and HERV-FRD, respectively, mediate intercellular fusion of trophoblast cells to form syncytiotrophoblast as well as prevent maternal immune attack against the developing embryo, thereby facilitating implantation of the embryo (Krone & Grange, 2010; Rote et al., 2004). It is estimated that 8% of the human genome consists of retroviral elements (Singh et al., 2009). A complete HERV sequence is composed of GAG, POL, and ENV genes flanked by two long terminal repeats (LTRs), similar to exogenous retroviruses such as human immunodeficiency virus (HIV) (Ahn & Kim, 2009). HERVs can be classified into over 20 families based on tRNA specificity of the primer binding site used to initiate reverse transcription; thus, HERV-K would use lysine and HERV-W tryptophan if they were replicating viruses. Increased HERV expression has been found under pathological conditions, particularly in cancer and inflammatory disease (Voisset et al., 2008). Unlike

most HERVs that harbor defective mutations, the HML-2 group of HERV-Ks has open reading frames that code for functional viral proteins, which may form noninfectious particles (Beimforde et al., 2008; Turner et al., 2001). We have demonstrated recently that expression of HERV-K correlates with ERK activation and p16 loss in human melanoma specimens, and that inhibition of MEK and CDK4, especially in combination, suppresses HERV-K expression (Li et al., 2010b), which suggests that HERV-K may mediate BRAF-MEK-ERK and p16-CDK4-RB signaling pathways in melanoma cells.

It is interesting to note that the growth characteristics of melanoma cells that can be modified by HERV-K activation (e.g., changes in cell shape, loss of melanin, anchorage-independent growth) (Serafino et al., 2009) overlap with those that can be blocked by suppression of the BRAF-MEK-ERK signaling pathway, especially with simultaneous restoration of p16 or inhibition of CDK4 (Li et al., 2010a; Rotolo et al., 2005; Zhao et al., 2008). This observation, together with the knowledge that aberrations in BRAF-MEK-ERK and p16-CDK4 pathways are early events and often co-exist during melanomagenesis, and the evidence that the RAF-MEK-ERK signaling pathway is required for the completion of HIV-1 reverse transcription (Mettling et al., 2008), prompted us to hypothesize that HERV-K is regulated by BRAF-MEK-ERK and p16-CDK4 pathways. We examined the expression of HERV-K GAG and ENV proteins, the active form of ERK (phospho-ERK, p-ERK), and p16 in a panel of human melanocytic specimens including 38 benign nevi and 34 melanomas. As summarized in Table 3, both HERV-K GAG and ENV proteins are largely cytoplasmic with occasional nuclear staining observed, whereas p-ERK is typically co-expressed in the nucleus and cytoplasm. Wild-type INK4A is expressed in both nucleus and cytoplasm, whereas mutant p16, if expressed, is either nuclear or cytoplasmic (Ghiorzo et al., 2004). HERV-K GAG cytoplasmic staining is over 10-fold more frequent in melanoma than in nevi (38% of melanomas vs. 3% of nevi, $p < 0.001$) (Table 3). Similarly, HERV-K ENV immunoreactivity is detected in the cytoplasm in 44% of melanomas and 11% of neval specimens (a 4-fold difference, $p = 0.003$). The nuclear staining of GAG and ENV are infrequently detected in both nevi and melanomas, and the differences do not reach statistical significance (Table 3). p-ERK staining is 5-fold more often positive in melanomas than in nevi (68% and 13%, respectively, $p < 0.001$). p16 staining, both in the cytoplasm and

Antigen	Positivity (%)		p value
	Nevi [n=38]	Melanoma [n=34]	
HERV-K GAG, cytoplasmic	3	38	$p < 0.001^{**}$
HERV-K GAG, nuclear	0	6	$p = 0.493$
HERV-K ENV, cytoplasmic	11	44	$p = 0.003^{**}$
HERV-K ENV, nuclear	8	12	$p = 0.7$
p-ERK, cytoplasmic and nuclear	13	68	$p < 0.001^{**}$
p16, cytoplasmic	79	50	$p = 0.014^*$
p16, nuclear	79	15	$p < 0.001^{**}$

Protein immunoreactivity, cytoplasmic or nuclear, is dichotomized as negative/decreased (<30% of cells staining positively) or positive ($\geq 30\%$ of cells staining positively).

** Difference is significant at the ≤ 0.01 level (2-tailed).

* Difference is significant at the ≤ 0.05 level (2-tailed).

Table 3. Expression of HERV-K GAG and ENV, p-ERK and p16 in neval and melanoma specimens

nucleus, is more frequently observed in nevi than in melanomas (cytoplasmic, 79% vs. 50%, $p = 0.014$; nuclear, 79% vs. 15%, $p < 0.001$) (Table 3). Figure 13 demonstrates representative staining patterns of HERV-K GAG, HERV-K ENV, p-ERK, and p16 in melanoma and neval specimens. Further analysis shows that the expression of HERV-K GAG in the cytoplasm of melanoma is positively correlated with p-ERK ($p = 0.005$) and negatively correlated with p16 cytoplasmic expression ($p = 0.012$) (Table 4). The expression of HERV-K ENV in the cytoplasm of melanomas is positively correlated with p-ERK ($p < 0.001$) and negatively correlated with p16 nuclear expression ($p = 0.046$) (Table 4).

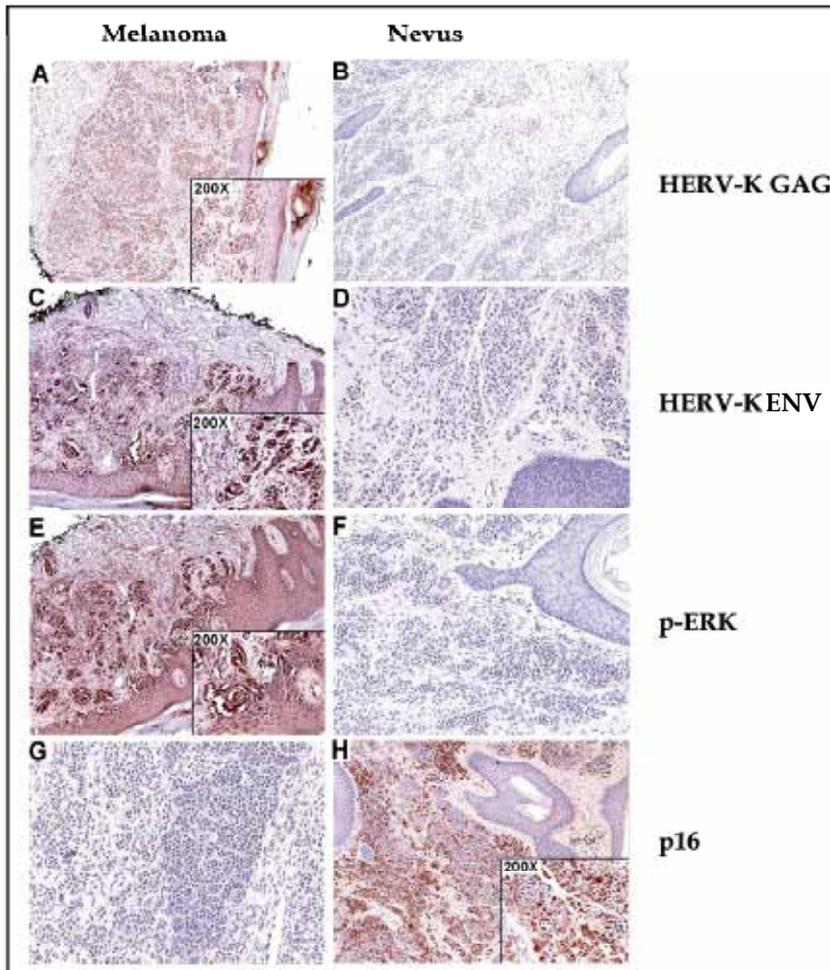


Fig. 13. Expression of HERV-K GAG and ENV, p-ERK and p16 in neval and melanoma specimens. Formalin-fixed and paraffin-embedded microscopic sections of melanoma (A, C, E, G) and nevus (B, D, F, H) are analyzed by immunohistochemical staining using HERV-K GAG (A, B), ENV (C, D), p-ERK (E, F), and p16 (G, H) specific antibodies. Shown is a representative staining pattern. HERV-K GAG and ENV are mostly detected in melanoma cells (A and C), but rarely expressed in neval cells (B and D). p-ERK is mainly detected in melanoma cells (E) but rarely found in neval cells (F). p16 is not as prominently expressed in melanoma (G) as in neval cells (H). Magnification: $\times 100$ and $\times 200$ (insets).

	HERV-K GAG cytoplasmic	HERV-K GAG nuclear	HERV-K ENV cytoplasmic	HERV-K ENV nuclear
p-ERK, cytoplasmic and nuclear	$p = 0.005^{**}$	$p = 0.093$	$p < 0.001^{**}$	$p = 0.2$
p16, cytoplasmic	$p = 0.012^*$	$p = 0.058$	$p = 0.114$	$p = 0.608$
p16, nuclear	$p = 0.123$	$p = 0.378$	$p = 0.046^*$	$p = 0.251$

Note: ** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Table 4. Associations of the expression of p-ERK, p16, and HERV-K GAG and HERV-K ENV

The expression of HERV-K has been reported in several melanoma cell lines (Buscher et al., 2006; Buscher et al., 2005; Muster et al., 2003; Serafino et al., 2009). We examined HERV-K in four melanoma cell lines (624Mel, A375, A101D, and OM431) that have constitutive activation of p-ERK and loss of wild-type p16 with corresponding hyper-phosphorylation of RB protein (Li et al., 2010a; Rotolo et al., 2005; Zhao et al., 2008). HERV-K expression is detected using a specific HERV-K ENV antibody, as described (Muster et al., 2003; Serafino et al., 2009), that recognizes a 37 Kd spliced transmembrane domain of ENV protein (Buscher et al., 2006; Buscher et al., 2005; Muster et al., 2003). HERV-K ENV protein is prominently expressed in 624Mel and A375 cells, weakly positive in A101D cells, but barely detectable in OM431 cells (Fig. 14A). We extracted total cellular RNA from all the four cell lines and performed conventional RT-PCR using specific HERV-K POL and ENV primers as described (Serafino et al., 2009). Gel electrophoresis of RT-PCR end-products shows similar levels of POL and ENV RNAs in the four cell lines, in contrast to the dramatic differences in the case of ENV protein (data not shown). Direct sequencing of RT-PCR amplicons and NCBI BLAST analysis show that expressed sequences share 96%-98% overall homology with Group N HERV-K (Romano et al., 2006) (data not shown), as reported in other melanoma cells (Hirschl et al., 2007).

Multiple endogenous and exogenous factors have been linked to the activation of HERVs including hormones, cytokines, and cytotoxic chemicals (Taruscio & Mantovani, 2004). To our knowledge, a direct association between ERK and p16-CDK4 pathways and HERV expression has not been reported previously. It has been shown that HERV-K sequences, as other host genes, are regulated by DNA methylation in the promoter/enhancer sequences located in the 5'-LTR regions (Lavie et al., 2005). Since RB protein, a downstream mediator of BRAF-MEK-ERK and p16-CDK4 signaling pathways, is a key regulator of DNA methylation (Montoya-Durango et al., 2009), it is conceivable that the observed association between HERV-K, p-ERK, and p16-CDK4 may act through RB, a notion that will surely prompt further investigation. It is worth noting that the four melanoma cell lines examined, 624Mel, A375, A101D, and OM431, all have BRAF T1799A mutation, constitutive activation of ERK, loss of wild-type p16, over-expression of phospho-RB protein (Rotolo et al., 2005), and have comparable levels of HERV-K RNA transcripts (not shown). However, only 624Mel and A375 cells express high levels of HERV-K ENV protein (Fig. 14A), suggesting that HERV-K ENV protein is regulated by mechanisms in addition to ERK and p16-CDK4 pathways. Alternatively, A101D and OM431 cells may harbor mutant HERV-Ks affecting EVE expression, a notion that needs to be further investigated.

We examined whether HERV-K ENV expression is suppressed by MEK and CDK4 inhibitors using established experimental conditions (including time course and dose-

response curve) for PD98059 and 219476, specific inhibitors of MEK and CDK4, respectively (Li et al., 2010a). As expected, treatment with PD98059 inhibited ERK phosphorylation in both 624Mel and A375 cells (Fig. 14B, lane 2). Phosphorylation of serine 780, a CDK4 target in the RB protein, is reduced by PD98059 and 219476, especially in combination, in both cell lines (Fig. 14B). Similarly, HERV-K ENV expression is inhibited by either PD98059 or 219476, especially when used in combination (Fig. 14B). The results are consistent with findings of the association between HERV-K expression and p-ERK and p16 in melanocytic specimens (Table 4).

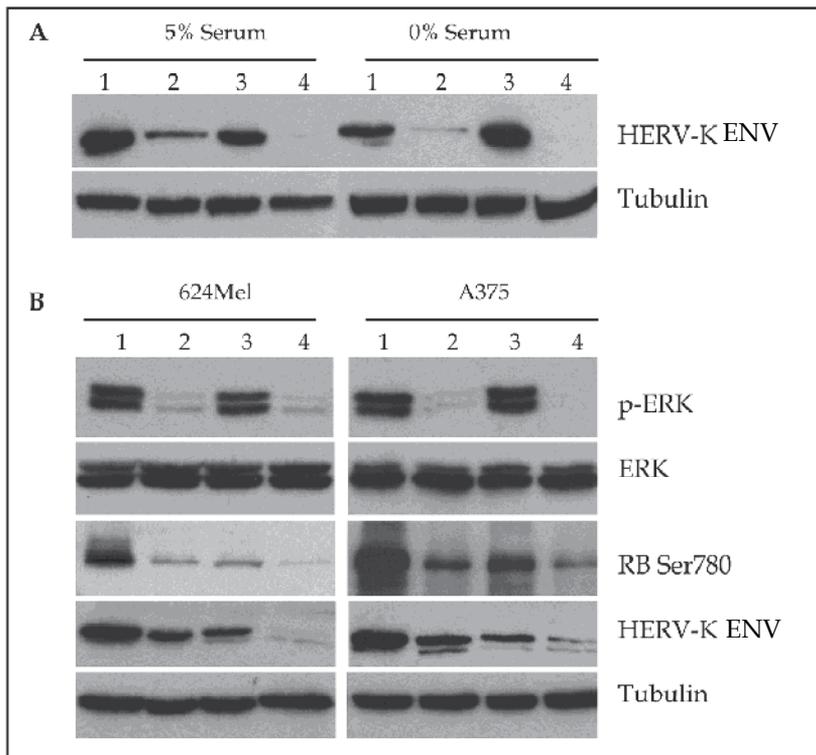


Fig. 14. MEK and CDK4 inhibitors suppress HERV-K ENV protein expression. (A) 624Mel (1), A101D (2), A375 (3), and OM431 (4) melanoma cells are cultured in DMEM with 5% serum or serum starved overnight, and cell lysates collected for Western blotting. (B) 624Mel and A375 melanoma cells are treated with solvent vehicle control (1), 25 µg MEK inhibitor PD98059 (2), 1 µg CDK4 inhibitor 219476 (3), and 25 µg PD98059 plus 1 µg 219476 (4) for 48 h under serum starvation, and cell lysates collected for Western blotting. Western blotting is performed using 50 µg total cell extracts and commercially available HERV-K ENV antibody that recognizes a 37 Kd spliced transmembrane domain of ENV protein. Tubulin is used as loading control.

HERVs have been implicated in the etiology of various diseases including cancer and chronic inflammation (Voisset et al., 2008), and emerging data support a role of HERV-K in melanomagenesis. For example, HERV-K is expressed in melanomas but not in normal melanocytes (Buscher et al., 2005; Muster et al., 2003), and specific inhibition of HERV-K by

RNAi suppresses melanoma cells *in vitro* and *in vivo* (Mangeny et al., 2005; Oricchio et al., 2007; Pothlichet et al., 2006; Serafino et al., 2009). There are several potential mechanisms to explain the role of HERV-Ks in melanomagenesis. First, HERV-K sequences may jump around by retro-transposition leading to mutagenesis and chromosomal abnormalities (Pothlichet et al., 2006; Tchenio & Heidmann, 1991) which may drive the evolution of aggressive clones. Second, HERV-K ENV, which is homologous to syncytin, may have fusogenic activity to mediate melanoma-melanoma and melanoma-target cell intercellular fusions and, therefore, can be the molecular link in the melanoma-normal cell fusion theory of metastasis (Carter, 2008; Pawelek & Chakraborty, 2008). Third, HERV-K proteins can be immunosuppressive (Mangeny et al., 2005) and may facilitate tumor progression by providing a critical survival/escape mechanism for tumor invasion and metastasis (Singh et al., 2009; Voisset et al., 2008), especially in the blood/lymph stream where circulating tumor cells are under attack by the host immune system.

HERV-K may prove to be a key mediator of BRAF-MEK-ERK and p16-CDK4-RB pathways during melanoma pathogenesis. Activation of BRAF/ERK is recently shown to drive chromosome abnormality and aneuploidy in melanocytes (Cui et al., 2010). It is conceivable that the effect may be mediated, at least partly, through HERV-K sequences that are capable of jumping around by retro-transposition leading to mutagenesis and chromosomal abnormalities (Pothlichet et al., 2006; Tchenio & Heidmann, 1991). It is possible that the observed growth promotion and anti-apoptotic effects of activated ERK and CDK4 (Li et al., 2010a; Zhao et al., 2008) can be mediated, at least in part, by HERV-K since HERV-K has been shown to directly affect melanoma cell proliferation, differentiation, and anchorage related survival (Oricchio et al., 2007; Serafino et al., 2009). Importantly, if HERV-K drives melanomagenesis downstream of the BRAF-MEK-ERK and p16/CDK4 pathways, when HERV-K is already turned on, cells may escape the inhibitory effects of therapies targeting BRAF-MEK-ERK and p16-CDK4. Triple therapies, such as simultaneous targeting of HERV-K, BRAF/MEK, and CDK4, may be necessary to produce more effective and long-lasting therapeutic effects than single or, as we have proposed previously, double inhibition of MEK-ERK and CDK4 (Li et al., 2010a; Zhao et al., 2008). This strategy is analogous to HIV “cocktail” therapy that disrupts HIV at different steps of replication and brought many AIDS patients from death to fairly normal and productive lives (Henkel, 1999).

10. Conclusion

Melanoma is the most lethal skin malignancy notorious for aggressive growth and therapeutic resistance. Multiple genetic and environmental factors have been linked to the development of melanoma. Approximately 60% of melanomas harbor mutations in v-raf murine sarcoma viral oncogene homolog B1 (BRAF) that lead to constitutive activation of the mitogen activated protein kinase/ERK kinase (MEK)-extracellular signal regulated kinase (ERK) signaling pathway. Results of the recent clinical trials with PLX4032, a specific inhibitor of mutant BRAF, have generated great excitement because approximately 80% of BRAF mutant metastatic melanomas regressed in response to PLX4032 treatment. Though this trial is considered a victory in the fight against melanomas, attention has been drawn to the fact that regressed tumors may resurge more aggressively within 8 months after the start of therapy indicating more research is needed to conquer melanoma. Most melanoma cells, but not nevi, have lost the expression of p16 inhibitor of CDK4 (INK4A), either through DNA mutation or promoter hypermethylation, resulting in a deregulated cyclin dependent

kinase 4 (CDK4)-retinoblastoma (RB) pathway. We found that simultaneous inhibition of mutant BRAF and expression of wild-type INK4A or combinatorial application of MEK and CDK4 inhibitors in melanoma cells significantly inhibits growth and enhances apoptosis. Our results support the hypothesis that simultaneous targeting of BRAF-MEK-ERK and p16-CDK4 pathways may be a promising strategy for melanoma treatment. Additionally, we found that HERV-K activation in melanoma cells is associated with BRAF-MEK-ERK and p16INK4A-CDK4 pathways. Our results should help the better understanding of BRAF-MEK-ERK and p16INK4A-CDK4 pathways, as well as HERV-K involvement in melanomagenesis and facilitate the design of novel management strategies. Further studies are warranted to examine the molecular mechanisms and biological consequences of these associations.

11. References

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Promising Experimental Therapies for Metastatic Melanoma

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1. Introduction

Advanced melanoma has the highest per-death loss of years of potential life expectancy second only to adult leukemia. The incidence of melanoma continues to rise worldwide at approximately 3% per year, and in 2009 there were an estimated 68,720 new cases in the United States and 8650 deaths (Balch et al. 2009). According to Surveillance Epidemiology and End Results (SEER) data, roughly 4% of melanomas are metastatic at time of diagnosis. Locally advanced or recurrent unresectable, and disseminated melanoma is notoriously unresponsive to standard treatment, and is associated with a dismal prognosis. Patients with metastatic melanoma have a median survival of 8 months and 2-year survival rates of 10% to 15%. (Tsao et al. 2004). Until March of 2011, only 2 drugs were approved by the Food and Drug Administration (FDA) in the United States (US) for metastatic melanoma: interleukin 2 (IL-2) and dacarbazine (ipilimumab was approved in March of 2011 and will be discussed in this chapter). Dacarbazine is an alkylating agent that in 1975 became the first chemotherapeutic drug approved by the US FDA for metastatic melanoma. Response rates for this regimen were 20% in a 30-year overview (Serrone et al. 2000), whereas more recent studies showed response rates in the 10-14% range (Bedikian et al. 2006; Chapman et al. 1999; Middleton et al. 2000). Temozolomide, an orally available congener of dacarbazine, was shown to be non-inferior in efficacy in comparison to dacarbazine in a randomized phase III trial (Middleton et al. 2000). Temozolomide had a similar objective response rate and overall survival compared to dacarbazine, and a significantly longer disease free survival. IL-2 was approved by the US FDA in 1998 for the treatment of metastatic melanoma. Single agent IL-2, when given in high dose intravenous boluses, leads to objective responses in 16% of patients, with complete responses in 6% and partial responses in 10%. Many patients maintained a complete response even after 7 years follow-up (Atkins et al. 2000). The toxicities of high dose IL-2 are substantial, including constitutional symptoms (fevers, chills, fatigue), hypotension, renal insufficiency, emesis, diarrhea and thrombocytopenia, and up to 2% of mortality is directly related to the treatment (Atkins et al. 2000). A systemic review of 41 randomized clinical trials including patients receiving various treatment schedules including biochemotherapy did not show improved progression-free survival (PFS) or overall survival (OS) (Eigentler et al. 2003). More recently, another meta-analysis of 18 trials involving nearly 2,500 patients with metastatic melanoma suggested an increase in response rate to biochemotherapy, but overall survival was not improved (Ives et al. 2007). Until today, biochemotherapy and combination chemotherapy built upon IL-2 and dacarbazine or temozolomide has not shown statistically

significant improvement in overall survival and cannot be regarded as standard therapeutic options for metastatic melanoma. Thus, more effective treatment modalities are urgently needed.

2. Agents targeting the immune system

2.1 CTLA-4

Increasing knowledge of T-cell regulation has uncovered potential immunologic targets for the treatment of melanoma. The interaction between antigen-presenting cells (APC) and T lymphocytes is crucial for inducing melanoma-specific T-cell responses. In addition to the antigen specific interaction between the HLA peptide complex on the APC and the T-cell receptor (TCR), several different co-stimulatory and co-inhibitory molecules modulate the T cell response. For instance, the T-cell surface molecule CD28 interacts with the B7 receptor on the APC to mediate a co-stimulatory signal (which is necessary in addition to the HLA-peptide-TCR interaction for efficient priming of the T cell), whereas the T-cell cytotoxic T-lymphocyte antigen (CTLA)-4 interacts with B7 to downregulate T-cell activation, acting as a natural "checkpoint" on the T-cell mediated immunologic response. Blocking interaction between CTLA-4 and B7 can overcome this checkpoint and enhance T-cell-mediated antitumor activity. This can be achieved by an anti-CTLA-4 monoclonal antibody (mAb). To date, two fully human anti-CTLA-4 mAb's have been developed for metastatic melanoma: tremelimumab (CP-675,206; Pfizer Inc., New York) and ipilimumab (MDX-010; Bristol-Myers, New Jersey).

In the initial phase I study, tremelimumab was relatively well tolerated and demonstrated encouraging clinical activity including several complete responses. However, in a randomized phase III study comparing single-agent tremelimumab with either dacarbazine or temozolomide in patients with advanced melanoma, tremelimumab failed to demonstrate a significant improvement in OS in comparison to standard chemotherapy (Ribas 2008). On the other hand, ipilimumab, in a randomized phase III clinical trial comparing ipilimumab alone versus gp100 vaccine alone to the combination of ipilimumab and gp100 vaccine, resulted in improved OS of nearly four months (median survival duration of 10.1 and 10.0 months in the ipilimumab arm and the combined arm, respectively, in comparison to 6.4 months in the vaccination alone (HR 0.66; 95% CI = 0.51-0.87; $p = .033$ and HR 0.68; 95% CI = 0.55-0.85; $p < .001$, respectively). A total of 676 HLA-A*0201-positive patients with unresectable stage III or IV melanoma, whose disease had progressed after one or more of the following therapeutic regimens: dacarbazine, temozolomide, fotemustine, carboplatin, or interleukin-2, were included in this study. This was the first randomized clinical trial that showed a statistically significant improvement in OS for metastatic melanoma (Hodi et al. 2010). This agent has now received FDA approval for use in metastatic melanoma.

The activity and side-effect profile of anti-CTLA-4 antibodies have several characteristics that reflect their immune-mediated mechanism of action. Objective responses observed in patients with metastatic melanoma with either tremelimumab or ipilimumab were seen in approximately 7-10% of patients. Remarkably, as much as 70% of responses were durable (Hodi et al. 2010; Ribas 2008). The unique pattern of response to CTLA-4 mAbs such as initial apparent progression of disease, even with emergence of new lesions, followed by regression and responses over the course of several months to years, has been seen with these agents (Hamid 2007). The recognition of this unusual kinetics of response has led to a

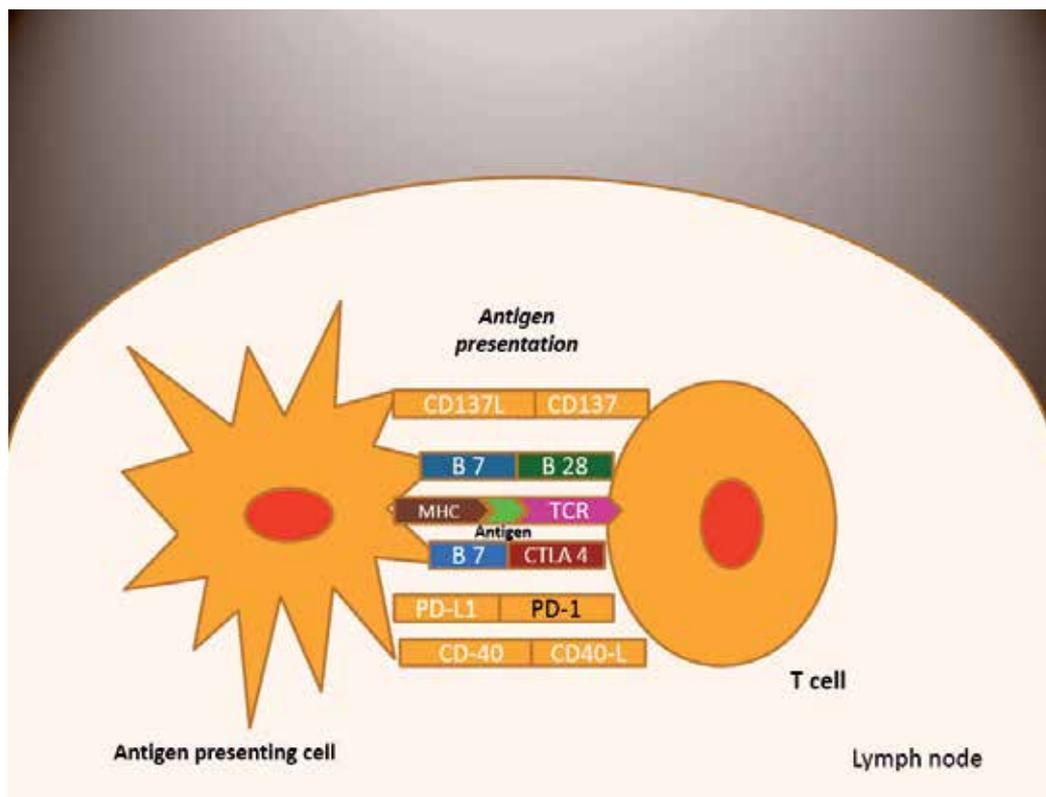


Fig. 1. T-cell response in melanoma: co-stimulatory and co-inhibitory signals. B7 proteins on antigen-presenting cells serve as ligands for both CD28 receptors and CTLA-4 inhibitory molecules on T cells. Upon T-cell receptor binding to antigen presented on MHCs, B7 proteins expressed on APCs can bind to CD28 or CTLA-4 receptors on T cells depending upon the precise expression patterns of the receptors and ligands and on the state of activation of the respective cells. Ligation of B7 to the CTLA-4 receptor results in inhibitory signals for T-cell activation and proliferation. Conversely, ligation of CD28 provides stimulatory signals to T cells leading to their activation. Interaction between PD-1 and PD-L1 results in inhibitory signals, whereas interaction between CD40 and CD40-L leads to T cell activation. Ag: antigen; CTLA-4: cytotoxic T-lymphocyte antigen 4; PD-1: programmed death 1; TCR: T-cell receptor.

proposal of immune-related response criteria (irRC) when evaluating treatment responses to anti-CTLA-4 mAb's (Wolchok JD 2009) (table 1). Immune related response criteria should replace currently accepted Response Evaluation Criteria in Solid Tumors (RECIST) for the evaluation of melanoma patients treated with immunotherapy. Indeed, it is possible that clinical efficacy of tremelimumab may have been missed in the phase III clinical trial owing to the failure to recognize that immune response criteria are needed for adequate response assessment.

The growing clinical experience with anti-CTLA-4 mAbs also identifies a constellation of autoimmune side effects, which have been designated "immune-related adverse events" or irAEs (Di Giacomo et al. 2010). These irAEs, including rash, autoimmune colitis, hepatitis,

	RECIST	irRC
New, measurable lesions (i.e., $\geq 5 \times 5$ mm)	Always represent PD	Incorporated into tumor burden
New, nonmeasurable lesions (i.e., $< 5 \times 5$ mm)	Always represent PD	Do not define progression (but preclude irCR)
Non-index lesions	Changes contribute to defining BOR of CR, PR, SD, and PD	Contribute to defining irCR (complete disappearance required)
CR	Disappearance of all lesions in two consecutive observations not less than 4 wk apart	Disappearance of all lesions in two consecutive observations not less than 4 wk apart
PR	$\geq 50\%$ decrease in SPD of all index lesions compared with baseline in two observations at least 4 wk apart, in absence of new lesions or unequivocal progression of non-index lesions	$\geq 50\%$ decrease in tumor burden compared with baseline in two observations at least 4 wk apart
SD	50% decrease in SPD compared with baseline cannot be established nor 25% increase compared with nadir, in absence of new lesions or unequivocal progression of non-index lesions	50% decrease in tumor burden compared with baseline cannot be established nor 25% increase compared with nadir
PD	At least 25% increase in SPD compared with nadir and/or unequivocal progression of non-index lesions and/or appearance of new lesions (at any single time point)	At least 25% increase in tumor burden compared with nadir (at any single time point) in two consecutive observations at least 4 wk apart

Table 1. Comparison between RECIST criteria and the irRC. BOR: best overall response; irCR: immune-related complete response; PD: progressive disease; PR: partial response; SD: stable disease; SPD: sum of the products of the two largest perpendicular diameters.

and less frequently hypophysitis and uveitis tend to be not harmful provided the clinician is aware of these unusual side effects, monitors the symptoms adequately, and knows when to intervene. IrAEs are thought to be a result of non-specific or cross-reactive tissue damage caused by activated T-cells. The most commonly reported rash is a macular and papular exanthema affecting trunk and extremities, which has been reported in 47–68 % of patients (O'Day et al. 2010; Weber et al. 2009; Wolchok et al. 2010). These dermatologic irAEs are generally mild and symptomatic relief is provided by antihistamines, while grade 3/4 autoimmune dermatitis is successfully treated with slowly tapered corticosteroids. At our institution, we reported the unique occurrence of hair depigmentation following therapy with CTLA-4 monoclonal antibody that correlated with durable clinical responses (figure 2). Six of 43 patients developed sudden hair depigmentation starting with the eyebrows, and continued to have either complete depigmentation of all body hair or the development of diffuse vitiligo. The median time to depigmentation was 10 months. All of these patients achieved partial response or complete response that sustained during the follow-up period of 24–36 months (Pavlick 2010).



Fig. 2. Progressive hair depigmentation due to ipilimumab over 3-month period. These photographs are published with patient's permission.

Diarrhea due to immune-mediated colitis is the most frequent gastrointestinal irAE; if untreated, it may lead to serious complications such as intestinal perforation (<1%) (Beck et al. 2006). Although grade 3–4 diarrhea is generally reversible with standard anti-

inflammatory therapies, experience suggests that patients with colitis treated with high-dose corticosteroids combined with mesalamine, and with seeming resolution of the adverse effects, can have an early recrudescence of the symptoms requiring either prolonged corticosteroid administration, tumor necrosis factor (TNF) blockade with infliximab, or prolonged bowel rest with total parenteral hyperalimentation (Yang et al. 2007). Other irAEs such as hepatitis, iridocyclitis and endocrinopathies including hypophysitis, hypothyroidism and adrenal insufficiency are uncommon but worth mentioning. Hepatitis generally presents as asymptomatic rise in the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In most cases, a delayed schedule of anti-CTLA 4 mAb leads to normalization of these levels. However, unlike other irAEs, hypophysitis, which occurs in 1.5% of patients treated with ipilimumab (Hodi et al. 2010) often does not improve with high-dose corticosteroids, and permanent hormone replacement therapy is required. Similarly the frequency of hypothyroidism and adrenal insufficiency is 1.5% each (Hodi et al. 2010). Interestingly, development and increasing severity of irAEs generally associate with tumor regression and with a prolonged time to relapse; nevertheless, this association is not absolute and patients without evidence of irAEs have also experienced significant clinical responses (Downey et al. 2007; Kahler and Hauschild 2010).

Melanoma is notorious for its propensity to metastasize to the brain. Up to 75% of patients develop this complication in autopsy series (de la Monte et al. 1983; Patel et al. 1978), and at least one third to one half of patients in clinical practice are found to have one or more brain metastases during the clinical course of their metastatic disease (Amer et al. 1978; Sampson et al. 1998). Brain metastasis portends a grave prognosis. The currently available treatment options for brain metastasis from melanoma include surgical and radiation therapies which may improve the outcome of selected patients with brain metastasis. The most favorable outcomes have been reported in patients who are candidates for stereotactic radiosurgery (Fife et al. 2004). Even though systemic treatment using cytotoxic agents, such as temozolomide in the US, often is employed in the setting of brain metastasis by virtue of the ability of temozolomide to cross the blood-brain barrier, its impact on overall survival remains unproven (Agarwala et al. 2004; Antonadou et al. 2002; Hwu et al. 2005). Up to date, no single treatment modality has been systematically tested in a randomized clinical trial to demonstrate an impact on disease-specific survival. Encouragingly, treatment with CTLA-4 mAb has resulted in durable control of brain metastases (Hodi et al. 2008b; Margolin et al. 2010; Scharz et al. 2010). While the phase III trials with ipilimumab excluded patients with prior or current brain metastasis, a parallel expanded access trial of ipilimumab enrolled patients who were later found to have small brain metastases that had not been treated with radiation. Among those patients, several were noted to have tumor regression in the brain. This unexpected observation led to a clinical trial initiated by the Cytokine Working Group specifically designed for melanoma patients with one or more brain metastases that did not require immediate surgery or radiotherapy. In this trial, patients were candidates for ipilimumab therapy if they had no systemic disease, untreated disease, or were no longer responding to a prior regimen for systemic disease. Brain metastases and extracranial disease were assessed separately, using standard criteria for tumor assessment based on the World Health Organization system and a composite global response status. The results of this trial, reported at the 2010 ASCO meeting, were encouraging. At week 12, for brain alone, the disease control rate was 21.5% (5 of 51 patients

achieved partial response, and 6 of 51 patients achieved stable disease); the duration of brain disease control ranged from 3 to 15 months (Lawrence 2010).

2.2 PD-1

The programmed death 1 (PD-1) receptor is a negative regulator of antigen-activated T cells (Fourcade et al. 2009). It bears homology to CTLA-4 but provides distinct co-inhibitory signals. The cytoplasmic domain of PD-1 contains 2 tyrosine signaling motifs that can attenuate the TCR/CD28 signal (Parry et al. 2005). There are two known ligands for PD-1: B7-H1/PD-L1 (hereafter PD-L1), the predominant mediator of PD-1-dependent immunosuppression, and B7-DC/PD-L2. PD-L1 is expressed by many tumors including melanoma, and its interaction with PD-1 resulted in tumor escape in experimental models (Iwai et al. 2002). Blockade of the PD-1: PD-L1 pathway in combination with prolonged antigen stimulation with melanoma cells augments the number of cytokine-producing, proliferating, and total NY-ESO-1-specific CD8+ T cells. In murine tumor models, the inhibition of PD-1: PD-L1 interactions was shown to reverse the generation of functional anergy and exhaustion in CD 8+ T cells (Chikuma et al. 2009), and to restore a considerable proportion of the effector activity of CD8+ T cells (Blank et al. 2004), which can reinstate their antitumor effects (Blank et al. 2004; Iwai et al. 2002; Strome et al. 2003). Current data indicate that there is a correlation between the degree of PD-L1 expression and the vertical growth of primary tumors in melanoma (Hino et al. 2010). Multivariate analysis demonstrated that PD-L1 expression is an independent prognostic factor for melanoma (Hino et al. 2010). Therefore, blockade of PD-1: PD-L1 interaction represents a rational strategy for cancer immunotherapy.

MDX-1106 (BMS-936558/ONO-4538) is a fully human immunoglobulin G4 (IgG4) mAb specific for PD-1. The drug binds PD-1 with high affinity and blocks its interaction with both PD-L1 and PD-L2. A phase I study of single agent MDX-1106 in refractory solid tumors was conducted in 39 patients with advanced metastatic non-small cell lung carcinoma (NSCLC), melanoma, castrate-resistant prostate cancer, renal cell carcinoma (RCC), or colorectal carcinoma (CRC). Although efficacy was not the primary endpoint of this phase I study, of the 39 treated patients, 1 durable complete response (CRC) and 2 partial responses (melanoma, RCC) were seen. Two additional patients (melanoma, NSCLC) had significant lesional tumor regressions, which did not meet criteria for PR. This study suggested a more benign immune-related toxicity profile for anti-PD-1 mAb than one seen associated with anti-CTLA-4 mAb. Only 1 patient with metastatic ocular melanoma developed grade 3 inflammatory colitis following five doses (1 mg/kg) administered over 8 months, which responded to steroids and infliximab, while grade 2 immune related adverse events occurred in 3 patients presenting with polyarticular arthropathies requiring oral steroids and hypothyroidism requiring hormone replacement. (Brahmer et al. 2010). Another phase I trial was conducted on 16 patients with metastatic disease including melanoma. Objective responses were documented in 37.5% of patients lasting 3-13+ months; half of the patients had melanoma and there were few irAEs (Sznol 2010).

2.3 Adoptive cell therapy

The transfer of tumor specific T cells has emerged as a promising therapeutic strategy for melanoma. Adoptive cell therapy (ACT) was pioneered at the Surgery Branch of the National Cancer Institute (NCI; Bethesda, MD), and to date, has shown some of the most

impressive response rates (up to 72%) in patients with metastatic melanoma. The most developed approach is based on the *ex vivo* selection of highly reactive, tumor-infiltrating lymphocytes (TIL), their activation and numerical expansion before reinfusion to the autologous tumor-bearing host (Dudley et al. 2005). Adoptive transfer after a nonmyeloablative conditioning regimen was shown to result in the persistent clonal repopulation of T-cells in patients with metastatic melanoma, with the transferred cells proliferating *in vivo*, displaying functional activity, and trafficking to tumor sites (Dudley et al. 2002).

By eliminating competition for endogenous serum cytokines, lymphodepletion may affect survival and proliferation of the adoptively transferred TIL. Data from animal models suggested that increased levels of lymphodepletion could improve ACT efficacy. In murine models, lymphodepletion seemed to enhance the antitumor effects of transferred T cells *in vivo* by several mechanisms including the elimination of suppressive CD4+, CD25+ T-regulatory lymphocytes, the elimination of cellular "sinks" for homeostatic cytokines such as IL-7 and IL-15, and the engagement of toll-like receptors on antigen-presenting cells after damage to the integrity of the gut epithelial lining (Antony et al. 2005; Gattinoni et al. 2005; Paulos et al. 2007). Cyclophosphamide and fludarabine have been employed for nonmyeloablative lymphodepletion. In a series of 35 patients, Dudley et al. showed that adoptive transfer of autologous TIL after nonmyeloablative but lymphodepleting chemotherapy with cyclophosphamide and fludarabine followed by high dose IL-2 resulted in objective responses in 51% of heavily pretreated patients with metastatic melanoma (Dudley et al. 2005). Further increasing intensity of host preparative lymphodepletion by adding total-body irradiation (TBI) of either 2 or 12 Gy prior to cell transfer in cyclophosphamide and fludarabine lymphodepleted patients was shown to generate response rates up to 52% and 72%, respectively. Of the 25 patients studied in the 12 Gy TBI arm, there were 4 complete responders and at least 2 substantial partial responders (Dudley et al. 2008).

The caveat of adoptive cell therapy is its complexity of several critical preparative steps that are both labor intensive and costly which may be the major obstacle to the widespread application of this approach. Up to 4 to 6 weeks of cell culture are required to obtain adequate numbers of reactive lymphocytes for adoptive transfer. Additionally, much akin to the limitation of high dose IL-2, only patients with an excellent functional status are able to withstand the intensity of adoptive cell therapy which may also involve TBI in addition to IL-2. On the other hand, as the data from the NCI suggest, such potent therapy in highly selected patients does have the potential to induce durable responses and a potential cure. To date, however, only small phase II clinical trials have been completed and no phase III study has been initiated. Meanwhile, further innovations beyond current TIL technology are underway. Current strategies seek to improve the yield of TIL cultures, to use vaccines to stimulate the transferred T cells *in vivo*, and to employ gene therapy using genetically engineered lymphocytes that express highly reactive T-cell receptors (TCRs) with specific anti-melanoma differentiation antigen activity (gp-100 or MART-1), produce IL-2 or other molecules promoting an effective immune response. The gene therapy approach is of particular interest because the anti-tumor effector T cells could be rapidly expanded for adoptive transfer. Furthermore, it could be of tremendous benefit to patients who do not have autologous reactive TIL available and would otherwise be ineligible for adoptive immunotherapy (Johnson et al. 2009; Yang et al. 2010). The objective response rates in trials employing gene therapy have been reported to be 20% to 45%, and durable tumor

regression at all disease sites including the brain has also been observed (Johnson et al. 2009; Morgan et al. 2006; Robbins et al. 2011).

3. Agents targeting oncogenic signaling pathways

The MAPK pathway (figure 3) has been of keen interest in melanoma in particular since Davies et al. first reported that 66% of melanomas harbor activating somatic missense mutations in the BRAF gene (V600E), leading to constitutive activation of this pathway (Davies et al. 2002; Meier et al. 2005). The RAF/MEK/ERK pathway is an important regulator of growth, survival and migration and it is constitutively activated in many human cancers (Fecher et al. 2008). Physiologically, the RAF/MEK/ERK signaling pathway is activated upon binding of extracellular ligands to growth factor receptors with intrinsic tyrosine kinase activity such as epidermal growth factor receptor (EGFR), c-Kit, platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor

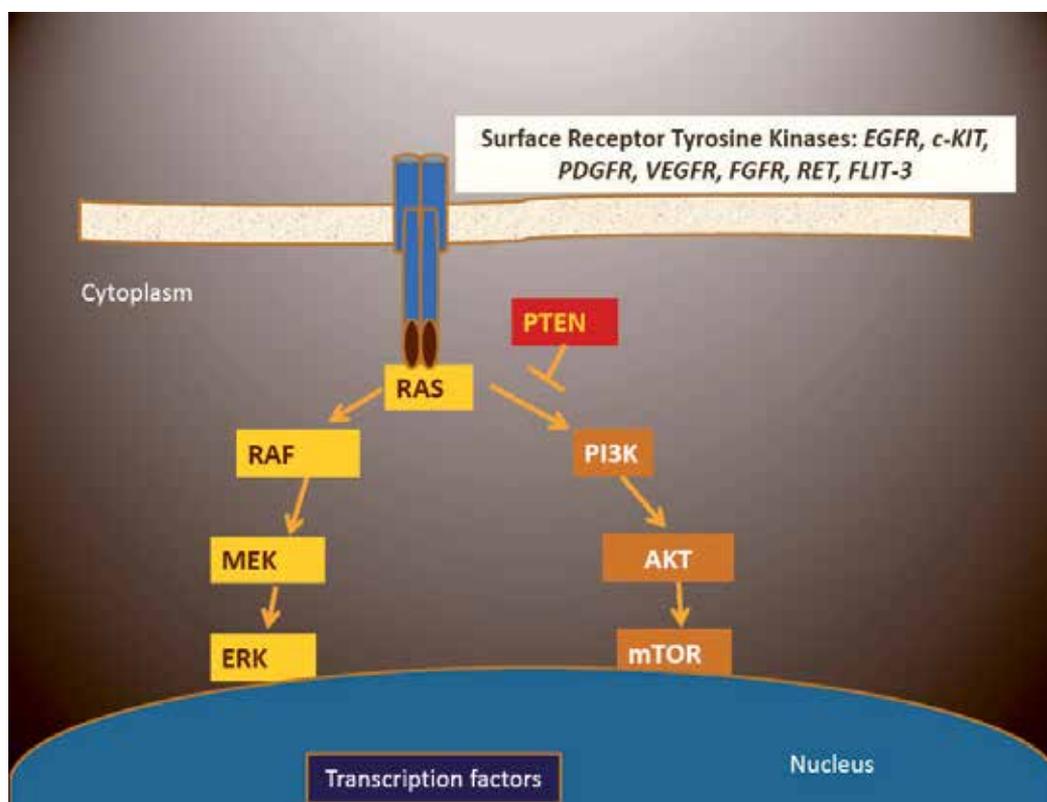


Fig. 3. Molecular targets in melanoma: the MAPK and PI3K pathways. Binding of growth factors to cell surface receptors induces their dimerization and activation of the MAPK tyrosine kinase cascade, which includes RAS, RAF, MEK and ERK. Activated (phosphorylated) ERK translocates into the nucleus, and phosphorylates transcription factors for genes involved in cell growth and proliferation. This pathway is constitutively activated in many human melanomas. An alternate pathway via PI3K, AKT, and mTOR is also activated in some melanomas.

(VEGFR), and fibroblast growth factor receptor (FGFR). Signaling through the RAF/MEK/ERK pathway eventually leads to the transcription of hundreds if not thousands of genes related to cellular proliferation, survival, apoptosis, tumor invasion, and motility (Fecher et al. 2008; Shields et al. 2007). Another kinase signaling pathway involves PI3K/AKT activating mTOR which promotes signaling to the nucleus and activates genes involved in cell growth and proliferation (Stahl et al. 2004). Studies have shown that a high proportion of melanomas carry alterations in the PI3K/AKT pathway (PTEN deletion or AKT amplification) independent of the BRAF^{V600E} mutation (Haluska et al. 2007; Vivanco and Sawyers 2002). These findings reveal a complex, interlinked network of cellular signaling pathways that contain several potentially synergistic therapeutic targets for metastatic melanoma.

3.1 RAF

Sorafenib (Bay 43-9006) was the first RAF inhibitor to enter early clinical trials. It is a small-molecule, multi-targeted tyrosine kinase inhibitor that blocks EGFR, c-Kit, Flt-3, PDGF and VEGF in addition to BRAF (Adnane et al. 2006; Wilhelm et al. 2004). Although Sorafenib does not have meaningful activity in melanoma as a single agent (Eisen et al. 2006; Ott et al. 2010), its use in combination with dacarbazine or temozolomide led to superior objective responses and progression-free survival compared to historical response rates to these agents (Amaravadi et al. 2009). However, in a phase III randomized trial (E2603) comparing carboplatin, paclitaxel and sorafenib versus carboplatin, paclitaxel and placebo in chemotherapy-naïve patients, the futility analysis demonstrated no benefit of the three drug combination compared to the two drug chemotherapy combination. In the PRISM study, the addition of Sorafenib to the combination of carboplatin and paclitaxel as second-line treatment after chemotherapy with dacarbazine or temozolomide failed to improve progression-free survival, response rate and time-to-disease progression in metastatic melanoma patients (Hauschild et al. 2009).

Since then, second generation selective RAF inhibitors have been developed and are actively tested in various clinical trials. RG7204 (Roche Pharmaceuticals, formerly PLX4032, Plexxikon) is an oral, highly selective inhibitor of the oncogenic V600E mutant BRAF kinase, which showed promising results in early clinical studies. In a dose-finding phase I trial, 11/16 (68%) of patients with mutant BRAF metastatic melanoma achieved PR and four patients had minor responses leading to a PFS of 8-9 months (Flaherty 2009). A dose extension phase I trial with 32 patients demonstrated an objective response rate of 81% (2 CRs, 24 PRs) (Flaherty et al. 2010). The median PFS among these patients was more than 7 months. RG7204 is generally well tolerated with rash, photosensitivity, arthralgia and nausea. Of note, 31% of patients developed grade 3 squamous cell carcinoma (SCC), keratoacanthoma (KA) type. The median time to the appearance of a cutaneous squamous cell carcinoma was 8 weeks with no reported involvement of other organs. Treatment with RG7204 was not interrupted by the appearance of these skin lesions and the majority of them were resected (Flaherty et al. 2010). On the basis of these promising results, a phase II trial (BRIM 2) is now fully accrued and a phase III trial (BRIM 3) met its primary end points with RG7204 improving both PFS and OS (Chapman, PB, *N Engl J Med.* 2011 Jun 30;364(26):2507-16. Epub 2011 Jun 5) comparing RG7204 to dacarbazine in untreated patients with BRAF V600E mutant metastatic melanoma.

GSK 2118436 is another oral, highly potent and selective inhibitor of the V600E/K/D mutant BRAF. In a phase I/II study, treatment with GSK 2118436 led to a decrease in FDG-PET metabolic uptake with 11/14 (79%) of melanoma patients showing a decrease from baseline (range -5 to -100%), and 18/30 (60%) patients demonstrated a > 20% tumor decrease by RECIST at first restaging (8-9 wks). GSK 2118436 showed good tolerability and low grade nausea, vomiting, fatigue, headaches and skin changes (including low grade SCC) were the main adverse effects (Kefford 2010). A phase II study of GSK2118436 as salvage therapy (NCT01153763), and a phase III study of GSK2118436 versus dacarbazine (NCT01227889) as front line therapy for mutant BRAF metastatic melanoma patients are underway.

Judging from the data available to date and the experience in our center, selective RAF inhibitors are relatively well tolerated, high grade adverse events are uncommon. Unique side effects associated with these novel drugs are the emergence of KA and SCC. These lesions can appear as early as 2 weeks after the initiation of therapy and are reported in 15-30% of the patients (Arnault et al. 2009; Flaherty et al. 2010). Histologically, KA is almost indistinguishable from a well differentiated SCC. In contrast to KA, SCC is a malignant lesion that does not regress spontaneously and has the potential for metastasis. The biologic mechanism and natural history of these lesions in the context of RAF inhibitors, in comparison to their spontaneous counterparts, are currently unknown (Arnault et al. 2009; Robert et al. 2010).

3.2 MEK

The serine/threonine tyrosine kinase MEK acts downstream from RAF in the RAF/MEK/ERK pathway; its inhibition is an attractive anticancer strategy as it has the potential to block upregulated signal transduction through this pathway, regardless of the upstream position of the oncogenic aberration. AZD6244 (ARRY-142886, Array BioPharma, AstraZeneca) is a selective MEK1/2 inhibitor that has shown preclinical activity (Yeh et al. 2007) and demonstrated clinical activity in a phase I study with a relatively benign toxicity profile (Adjei et al. 2008). These results led to a randomized phase II trial comparing AZD6244 versus temozolomide in chemotherapy naïve advanced melanoma patients (Dummer 2008). A total of 200 patients were enrolled with 104 and 96 patients randomized to AZD6244 and temozolomide, respectively; those who progressed on temozolomide could crossover to AZD6244. Results showed a trend toward OS in patients with BRAF mutations in the AZD6244 arm (Dummer 2008). At the 2010 ASCO annual meeting, the early results of a phase I study with AZD6244 in combination with docetaxel, dacarbazine or temsirolimus suggests the presence of BRAF mutation was significantly associated with clinical responses and increased time to progression. (Patel 2010). Several phase II studies of AZD6244 are underway for advanced melanoma patients with BRAF mutations as front line therapy: for treatment-naïve patients versus temozolomide (NCT00338130), in combination with dacarbazine versus dacarbazine alone (NCT00936221), as well as salvage therapy (NCT00866177). GSK1120212 is another potent and selective inhibitor of the MEK1/2 enzymes with promising anti-tumor activity in a phase I clinical trial, resulting in response rate > 70% in advanced melanoma patients with known BRAF mutations, including one patient who was previously treated with PLX4032 (Infante 2010). This drug is currently investigated in phase II and III clinical trials for advanced BRAF mutant melanoma patients who were either previously treated with a BRAF inhibitor or not. (NCT01245062, NCT01037127).

Although targeting the MAPK pathway is a promising new therapeutic approach for the treatment of melanoma, and treatment with selective BRAF and MEK inhibitors can induce high response rates, the limited duration of these responses in most patients, most likely because of emerging resistance to these inhibitors represents a significant clinical challenge. Molecular redundancy, in part due to the existence of RAF isoforms and signaling through alternative oncogenic pathways, such as PI3K/AKT/mTOR pathway (Jiang et al. 2011; Paraiso et al. 2010), receptor tyrosine kinase (PDGFR β)-dependent pathway (Nazarian et al. 2010) and COT (MAP3K8) (Johannessen et al. 2010), may provide the melanoma cells' escape mechanisms to specific pathway inhibitors and underscore their ability to adapt to pharmacological challenges (Jiang et al. 2011; Paraiso et al. 2010). In preclinical models, it has been reported that acquired resistance of melanoma cells to the BRAF inhibitors was associated with rebound activation of the RAF/MEK/ERK pathway (Paraiso et al. 2010). In line with this finding, activating signals to downstream MEK/ERK has been shown to switch to ARAF (Villanueva et al. 2010) or CRAF (Montagut et al. 2008; Villanueva et al. 2010) via N-RAS upregulation (Nazarian et al. 2010) to overcome the effect of BRAF inhibition. Moreover, the majority of melanoma cells harboring the BRAF V600E mutation retained the wild-type BRAF allele which could be rescued from the effects of BRAF knock-down by extracellular growth factors such as basic fibroblast growth factor, hepatocyte growth factor or endothelin-1 (Christensen and Guldborg 2005).

3.3 C-Kit

Imatinib is a selective tyrosine kinase inhibitor with multiple targets, including c-Kit and PDGR receptors, and has shown to be highly efficacious in chronic myelogenous leukemia and GIST tumors (Heinrich et al. 2000). Initial phase II trials with this agent in melanoma were disappointing with no objective responses (Ugurel et al. 2005; Wyman et al. 2006). However, gain of function mutations, gene amplifications and over-expression of c-kit were subsequently reported in 30-40% of mucosal, acral and cutaneous melanomas with chronic sun damage (Curtin et al. 2006). Impressive tumor regression was documented in a patient with mucosal melanoma who carried a mutation in the juxtamembranous domain of c-Kit (exon 11) in response to single agent imatinib (Hodi et al. 2008a). Moreover, preclinical studies showed sensitivity of c-Kit mutant mucosal melanoma, providing a rationale for the use of imatinib in this melanoma type. Preliminary results of a phase II trial evaluating the effect of imatinib in patients with metastatic melanoma with c-kit aberrations were presented at ASCO 2009. Over 30% of patients achieved a response (complete and partial response), whereas 50% had disease stability (Carvajal 2009). Another phase I/II study to define safety and efficacy of imatinib in combination with temozolomide in patients with unresectable, stage III/IV melanoma is currently underway. After the data on c-kit alterations became available when the trial was already in progress, patients with mucosal, acral and chronic sun damage melanomas were preferentially enrolled in the phase II part of the study. Early results of the trial were presented at ASCO 2008 (Fecher 2008). Of the 23 patients treated, 16 had been enrolled in phase I and 7 in phase II. The combination was well tolerated and demonstrated anti-tumor activity in melanoma. Of the 7 patients treated in the phase II trial, 1 patient had a CR and 6 had PRs (Fecher 2008). Phase II studies with a second generation c-kit inhibitor (nilotinib) in first or second line therapies for advanced melanoma with c-Kit mutation or amplification are ongoing (NCT01168050, NCT01099514). In addition, the multi-targeted receptor tyrosine kinase inhibitor sunitinib has shown potential efficacy in patients with c-kit mutated melanoma in an early phase clinical trial (Minor 2010).

3.4 MTOR

Resistance of mutant BRAF melanoma cells to RAF/MEK inhibition may also be due to activation of other survival signaling pathways such as the PI3K/AKT/mTOR pathway (figure 3) resulting in melanoma development and progression (Shao and Aplin 2010; Stahl et al. 2004; Villanueva et al. 2010). Recent reports suggested a significant correlation of increased PI3K-AKT activity with resistance to RAF/MEK inhibitors in melanoma (Gopal et al. 2010; Villanueva et al. 2010) and that inhibition of the PI3K/AKT/mTOR pathway could suppress MEK inhibitor-induced activation of AKT and resulted in synergistic cell killing with a MEK inhibitor (AZD6244) (Gopal et al. 2010). This provides a rationale for combinatorial therapy leading to dual inhibition of both RAF/MEK/ERK and PI3K/AKT/mTOR pathways. A phase II trial of the mTOR inhibitor temsirolimus (CCI-779) combined with the MEK inhibitor AZD6244 is currently recruiting treatment-naïve patients with BRAF mutant advanced melanoma (NCT01166126). In addition, inhibition of the PI3K/AKT/mTOR signaling pathway was found to sensitize melanoma cells to chemotherapeutic agents such as cisplatin and temozolomide *in vitro* as evidenced by enhanced apoptosis and suppressed invasive tumor growth (Sinnberg et al. 2009). A Phase II study of the mTOR inhibitor everolimus in combination with paclitaxel and carboplatin in patients with metastatic melanoma is in progress (NCT01014351).

4. Other targeted drugs currently in clinical development

4.1 PARP Inhibitors

Poly-ADP ribose polymerase (PARP) is a key enzyme in DNA repair. ADP-ribosylation is involved in DNA excision repair and inhibition of the enzyme enhances the cytotoxicity of DNA damaging agents (Durkacz et al. 1980). PARP inhibition in BRCA-deficient breast cancers has shown a favorable therapeutic index. In a phase II trial, olaparib, a novel poly-ADP ribose polymerase (PARP) inhibitor, achieved an objective response rate of 41% in women with BRCA1 or BRCA2 mutations and advanced breast cancer (Tutt et al. 2010). In melanoma, PARP-1 expression has been shown to correlate with tumor thickness (Staibano et al. 2005), a poor prognostic factor for melanoma, and over-expression of PARP-1 is correlated with recurrence and/or progression of the disease (Csete et al. 2009), suggesting that it potentially is as a promising new therapeutic target. AG014699 is the first PARP inhibitor to enter a clinical trial and was studied in combination with temozolomide in a phase II study with 40 chemotherapy-naïve metastatic melanoma patients. Of the 20 evaluable patients, there were 4 confirmed PRs and 4 SDs (Plummer 2006).

4.2 Antibody-drug conjugate

Glycoprotein NMB (GPNMB) is over-expressed in a variety of cancers including melanoma. CR011-vcMMAE is an antibody-drug conjugate comprised of a fully-human monoclonal antibody directed at the extracellular domain of GPNMB linked to a potent tubulin destabilizing agent, monomethyl auristatin E (MMAE). The enzyme-sensitive linker is designed to be stable in the bloodstream and to release MMAE inside tumor cells, resulting in cancer cell death. A phase I/II study of CR011-vcMMAE in patients with advanced, pretreated melanoma has completed accrual (NCT00412828). At the ASCO annual meeting in 2010, CR-vcMMAE at 1.88mg/kg, when given every three weeks, was reported to produce an objective response rate of 15% (Hamid 2010).

4.3 Antibodies against integrins

Integrins of the α_v family, such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$, are implicated in tumor-induced angiogenesis and are thought to play a role in tumor growth. CNTO 95 is a fully human monoclonal antibody directed against α_v Integrins, which has demonstrated anti-tumor and anti-angiogenic activities in animal models (Trikha et al. 2004). Results of a phase I study to assess safety and pharmacokinetics of CNTO 95 in patients with advanced refractory solid tumors demonstrated good tolerability (Jayson 2004). A phase I/II multicenter, randomized and double-blind study to assess the safety and efficacy of CNTO 95, alone and in combination with dacarbazine, in stage IV melanoma patients has been completed and result pending publication (NCT00246012). Another phase I trial of CNTO 95 in patients with metastatic melanoma and angiosarcoma recently demonstrated a manageable toxicity profile, and encouragingly, 2 of 18 patients with melanoma, who had failed several therapeutic regimens, sustained stable disease 5 and 7 months, respectively (O'Day et al. 2011).

5. Conclusion

Understanding the molecular biology of melanoma and unraveling of several signaling pathways over the last few years, as well as advances in immunology have led to the development of a number of promising novel therapeutic treatment options for metastatic melanoma. Immune-checkpoint blockade with ipilimumab has demonstrated increased overall survival in a randomized phase III clinical study (Hodi et al. 2010). Other immune-related approaches, such as anti-PD1/PD-L1 and adoptive cell therapy, are on the horizon. The identification of the BRAF V600E mutation led to the development of new agents that specifically block the MAPK pathway. Improved PFS and OS as a result of treatment with the V600E mutant BRAF kinase inhibitor RG7204 in BRIM 3 have been reported. Encouraging response rates with imatinib therapy in patients with mucosal, acral, and sun-damaged skin melanomas that harbor mutations or amplification in the c-kit gene have also been documented. These results herald the era of individualized therapies based on specific tumor genotypes, of which scientifically thoughtful combinations will usher in a new standard of care for metastatic melanoma in the near future.

6. References

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Part 4

Electrochemotherapy

The Role of Electrochemotherapy in the Treatment of Malignant Melanoma

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1. Introduction

About 68,130 new melanomas will be diagnosed in the United States during 2010 (38,870 men; 29,260 women) and of those 8,700 people will die of the disease (5,670 men; 3,030 women). The death rate has been dropping since the 1990s for those younger than 50, but has remained stable or is rising for older individuals. However, the incidence of melanoma has been increasing for at least 30 years, and this trend has become more pronounced in young white females and in older white men¹.

Malignant Melanoma is the seventh most common type of cancer, but it is the first cause of death from cutaneous skin cancers². It has been estimated that the lifetime risk of developing malignant melanoma is 2% (1 in 50) for Caucasians, 0.1% (1 in 1,000) for those of black descent, and 0.5% (1 in 200) for Hispanics³.

When melanoma is detected in advanced stages, it carries a dismal prognosis, with a mean survival of about 8 months and a 5-year survival as low as 5%⁴⁻⁶. The disease spreads both by the lymphogenous and the haematogenous routes and can metastasize to virtually any organ in the body. When secondary tumours emerge, these usually follow a sequential pattern to regional lymph node basins, followed by distant sites including skin, subcutaneous tissue, lung, liver, brain, bone and other viscera⁵⁻⁶. Local recurrence, in-transit metastases and satellitosis (cutaneous metastases within 2 cm of original lesion) represent the same dissemination process⁴ in the dermal lymphatics. When the patients present with cutaneous metastases, they are considered to have stage IIIB disease⁷. Cutaneous metastases occur in 2-20% of patients, depending on tumor thickness^{6, 8-9} and can occur either during the early or late phase of the disease¹⁰. In many instances they can represent the first site of recurrence after surgical excision of the primary tumor¹¹. The majority (70-80%) of recurrences are diagnosed within the first 3 years of initial diagnosis, and the median time to the presence of in-transit disease could range between 3 to 16 months⁴. The recurrences present as local or in-transit disease in 20-28%, regional disease in 26-60% and as distant metastases in 15-50% of patients. Even though local recurrence is

not yet considered stage IV disease, the prognosis is poor with 5 year survival rates of less than 50%^{9,12}. The risk of developing metastases may be predicted at excision of the primary lesion and determinants include, increasing thickness of the tumour, the anatomical location, histological subtype and gender¹³. In general tumours of the trunk, non cutaneous and subungual regions behave more aggressively than those of the extremity¹⁴⁻¹⁶.

2. Treatment limitations for recurrent or in-transit melanoma

Treating extensive cutaneous/subcutaneous nodules or in-transit disease is a clinical challenge because of common unresectability and relative insensitivity to conventional systemic therapies⁶. These recurrences provide a significant psychological burden for patients whose quality of life is negatively affected by the symptoms caused by the tumor, such as pain, bleeding, ulceration, and malodorous discharge^{4, 17-18}. Many patients suffering from in-transit disease also manifest with systemic disease, within 6-13 months of the onset of the local lesions. Their life expectancy is foreshortened and is determined by factors such as the burden of the loco-regional disease, the interval from initial treatment and their immune status⁴.

The aim of the treatment should be the elimination of both local and systemic disease with the benefit of improved life expectancy and quality of life, while minimizing toxicities or deformities. It is important to note that metastatic cells have disseminated prior to surgical intervention, outlining the critical need for the development of adjuvant therapeutic strategies¹⁹. Thus the treatment of unresectable and in-transit disease is both systemic and loco/regional. The options include surgery, which is the most effective method for limited recurrent or in-transit disease, systemic chemotherapy^{12,21}, radiation therapy²², cryosurgery²³; carbon dioxide laser ablation,^{12,24-26} intralesional therapies^{12,21} with the Bacille Calmette-Guerin vaccine²⁷, TNFerade a non replicant adenovirus complex, expressing the Tumor Necrosis Factor alpha (TNF- α) gene (hTNF- α cDNA)^{12,28}, and Interleukin-2²⁹, Cell Vaccine and Immunotherapy³⁰⁻³³, Interferon alpha³⁴⁻³⁶, Regional therapies such as hyperthermic isolated limb perfusion (HILP) and Isolated limb infusion (ILP)³⁷, Novel Molecular Therapies with c-Kit inhibitors (Imatinib), C-RAF-inhibitors (Sorafenib)³⁸, and blocking B-RAF regulation of mitogen-activated protein kinase pathway (MAPK)³⁸. In more recent years electroporation with anticancer drugs which is termed electrochemotherapy (ECT) has been successfully applied for local tumour control^{17, 19, 39}. In general malignant melanoma is refractory to systemic treatments and survival after treatment of regional cutaneous metastases has been reported to be between 20-28% at 10 years^{6,9,40}. However all current treatment modalities have individual limitations and variable response rates^{4, 12, 19, 41-42}.

3. Electrochemotherapy (ECT)

Definition and physiological effect:

The cell membrane represents a physical barrier to the intracellular transfer of hydrophilic drugs, macromolecules, nucleotides and peptides. The movement of polar and hydrophilic molecules across the membrane is restricted by the phospholipid bilayer¹⁸. Studies suggest that exposure to sufficiently strong and long electrical fields, could rearrange the lipid bilayer, by reorienting the hydrophilic heads in pore-like fashion, while the hydrophobic

tails are embedded within the plasma membrane. This porosity is transient and reversible if short duration high amplitude square wave electrical pulses are optimized.¹⁸

Electroporation (EP) of tumour nodules allow the permeation, into the cancer cells, of poorly permeable antineoplastic drugs, such as bleomycin or cisplatin which are given either systemically or intratumorally (IT)^{18, 39, 43-46}

The temporary permeability of the cell membrane caused by the electric pulses facilitates a potent localized effect and magnifies the drugs cytotoxicity by orders of magnitude ^{18, 39, 46-50}.

Types of Drugs

After several studies of different cytotoxic drugs, two have been identified as the best candidates for ECT: Bleomycin and Cisplatin ^{17, 45}. Of importance in these studies EP or the drugs on their own do not influence the growth of tumours, yet their combination have potent tumoricidal effects^{18, 51-53} (See Figure 1). An advantage of ECT is that it requires much lower doses of the cytotoxic drug for optimal effects than those usually used for systemic treatments. In addition there is little in the way of collateral damage or complications, since the the cell killing is confined to the tissues affected by the electric field. Bleomycin intercalates into the cellular chromatin causing single and double stranded breaks in DNA resulting in a mitotic cell death by pseudoapoptosis. (Figure 1 B,C & D). On the other hand, Cisplatin creates an apoptosis effect on the cell ^{18, 46}. This cytolytic activity is potentiated more than 1,000 fold for Bleomycin and 100 fold for Cisplatin by the addition of EP ^{12, 19, 48-49}.

The Vascular Lock

The electric pulses produce a transient state of hypoperfusion by local reflex vasoconstriction at the arteriolar level (lasting 1-2 minutes) and a phase of interstitial edema (that resolves with membrane resealing). However the effect may last longer (12 hours to 5 days) in rapidly dividing tumor cells and is more prominent in tumours with a less mature endothelial lining and higher interstitial pressure. This phenomenon mediated by the sympathetic nervous system is termed the "vascular lock" and it has implications for timing of drug administration¹⁸⁻¹⁹. After application of the electric current, there is a retention of drugs already in the tumour but there is also an impairment of entry of drugs from the circulation. Thus when the cytotoxic drugs are administered systemically sufficient time should be allowed to achieve optimal intratumoral drug concentration prior to application of EP. ECT produces other vascular influences, which are believed to be secondary to a reduction in local angiogenic factor production, such as endothelial cell destruction and neovascular reorganization ^{46, 54-55}. The combined vascular influences have been successfully exploited in the treatment of bleeding melanomas ⁵⁶⁻⁵⁷- which may be a difficult to manage problem, sometimes refractory and fatal, in patients with unresectable disease.

4. Technique of electrochemotherapy

1. The Equipment

The electric pulses may be applied to the tumors either by plate electrodes on the skin surface or by needle electrodes inserted into the lesion (figure2). The electric field distribution is determined by the geometry of the electrodes. Regardless of the type of electrode the electric field is highest around and between the electrodes ¹⁷⁻¹⁸. The current pulse generators in use are the Cliniporator (IGEA, Carpi, Italy) (FIG 2) and the Medpulsor™ (Inovio Biomedical Corporation, CA, USA). It appears that plate electrodes are

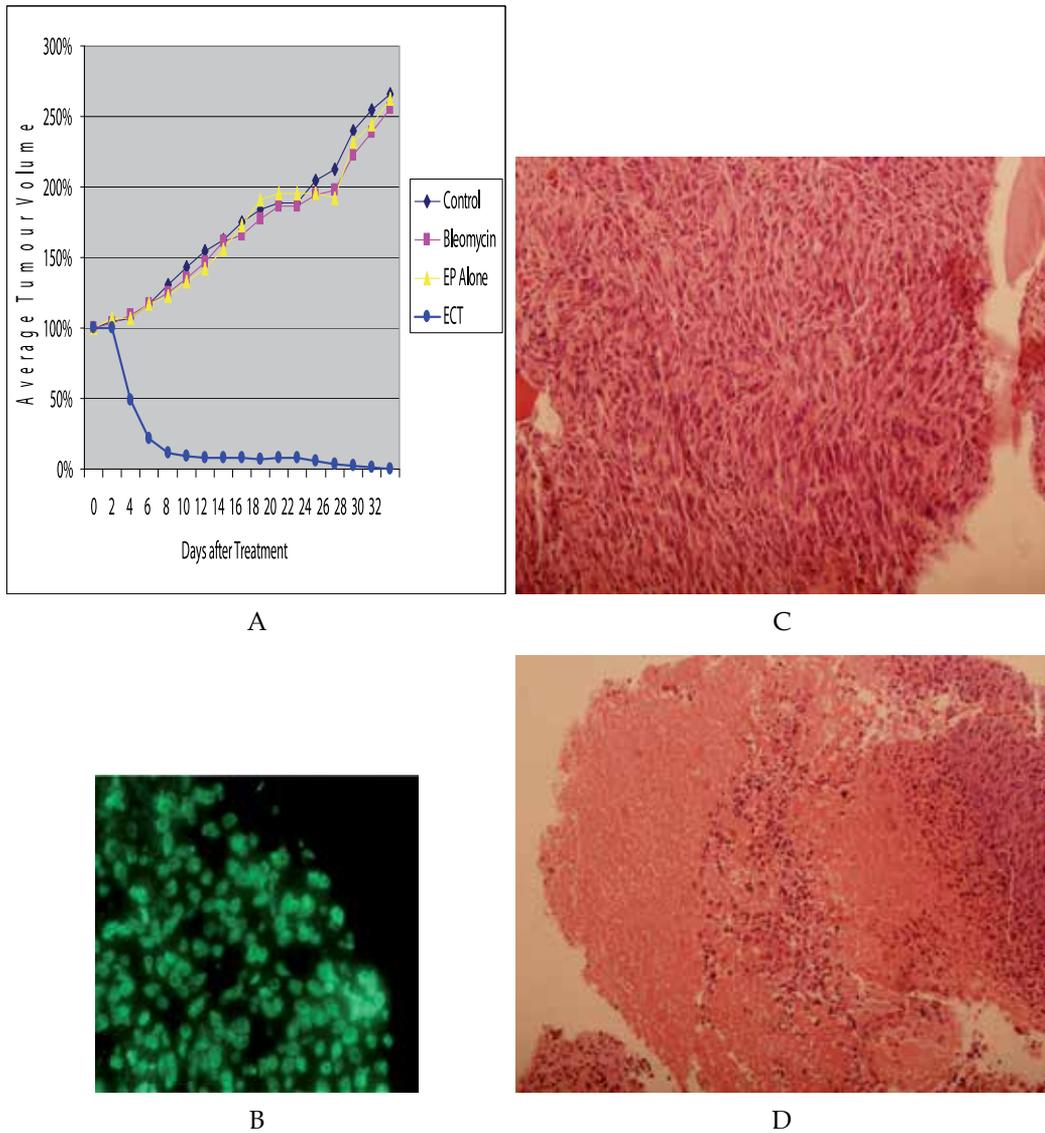


Fig. 1. A. Growth curves of experimental cancers in mice, which demonstrate that electropermeabilisation or intratumoural bleomycin alone, have no influence on tumour growth but when used together completely ablate the tumour. B) Positive Tunel stain 48 hrs post ECT indicating tumor cell death by apoptosis. C & D Tumour before showing normal cellularity and D 48 hrs post ECT showing regions of denudation

more suitable for use in superficial skin lesions, while needle electrodes are used for deeper seated lesions, such as exophytic and thick lesions (maximum depth 3 cm)⁵². A disadvantage of plate electrodes over the needle type is the potential skin damage that may be generated by the higher impedance/resistance of the skin, especially when treating larger affected areas^{19, 58}. Care must be taken to avoid inserting the needle electrodes into the healthy tissue surrounding the tumors, which may also result in local subcutaneous burns³⁹. There are three types of electrodes in common usage (FIG 2). Type I electrodes consist of two parallel stainless-steel plate electrodes, used for superficial lesions and do not penetrate the skin. Type II electrodes are used for smaller lesions and consist of two rows of eight needles with 4 mm distance between them, while Type III electrodes are recommended for larger lesions (>1cm), with the needles in a hexagonal configuration. The needles are inserted encircling the tumor and down to the subcutaneous tissue, slightly deeper than tumor depth^{17, 19, 52, 59}. It is recommended to elevate or tent the skin at the time of delivering the electric current, if the electrodes are to be inserted in a superficial or shallow subcutaneous areas such as near the knees, tibial tuberosities, the scalp, or close to any other osseous structures⁵²

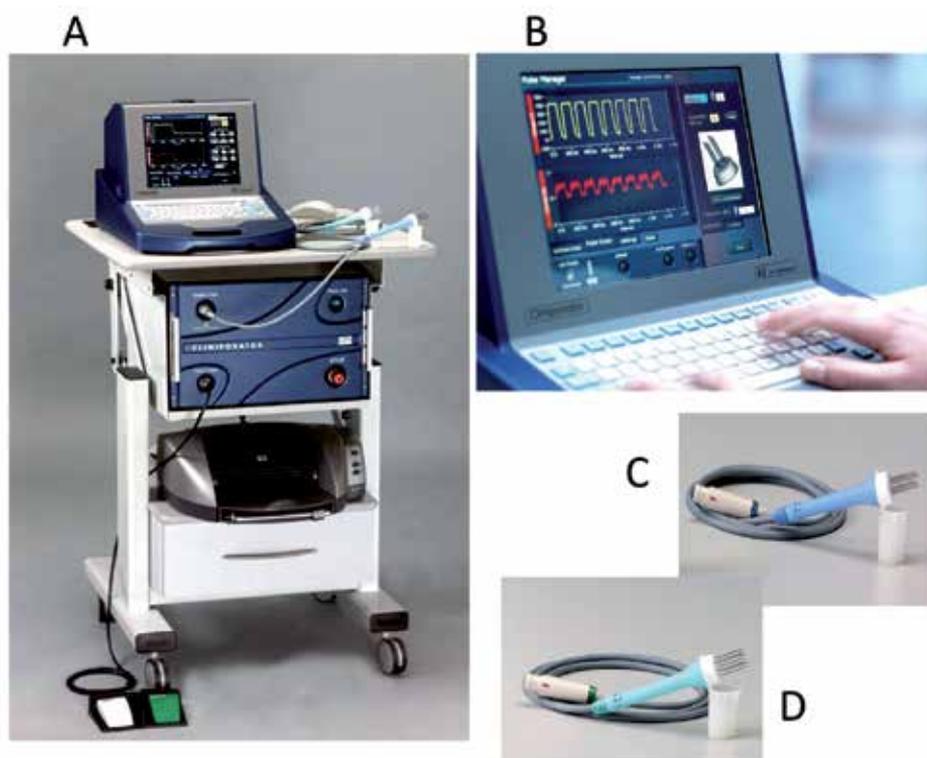


Fig. 2. (A) The ECT pulse generator. B) Note an entrainment of 8 square wave pulses. C) Electrodes type1 (plates) and (D) type 3 (Needle array).

2. Anesthesia

The procedure can be performed in the outpatient or ambulatory setting, under local anesthesia in association with conscious sedation. General anesthesia may be preferred for larger tumors, or tumors located in prior irradiated or fibrotic tissues where infiltration of local anesthetic may be painful and less likely to diffuse and thus achieve adequate pain control and for those located too close to vascular or bony structures. The dose of local anesthetic should be recorded and should not exceed the maximum allowed per body weight for Lidocaine without epinephrin (3 mg/kg)^{52, 59-60}. In addition, a mixture of O₂/air with FiO₂ of 40 % is administered to the patients during conscious sedation.

3. The Electric Current

Permeabilization occurs in the cell when the internal transmembrane potential has surpassed the critical value between 200-300mV. The extent of the electroporative effect depends on the number and duration of the electric pulses^{18,55}. Two pulse parameters have been evaluated - exponentially decaying pulses and square wave pulses. Of the two, square wave pulses are preferred as they permit independent control of the length and amplitude. The pulse parameters selected for treatment depend on the type of electrode^{18, 52}.

Pulse parameters

Ideally for Type I (plate) electrodes, pulse parameters of 8 square waves with amplitude of 1300 V/cm, duration of 100 μs, and frequency of 1Hz are used. The current should be delivered in two perpendicular directions.

For type II (needle) electrodes the voltage amplitude may be reduced to 1000V/cm. The pulses are administered simultaneously between the needle pairs.

For Type III, the needles are positioned hexagonally and 96 pulses are given together (12 pairs x 8 pulses) at a frequency of 5 kHz⁶¹.

Due to the already described vascular lock phenomenon, it is recommended that the electric current is applied between 8 to 28 minutes after IV administration of the drug⁶², or immediately (within 2-10 minutes) after intratumoral administration^{19, 59, 63-64}.

4. Drug Dosage Recommendations

Initial studies compared the routes of administration of Bleomycin suggested advantages for the intralesional over intravenous administration (77% vs. 45 % complete responses)⁴⁵ but later the prospective multi-institutional ESOPE (European Standard Operating Procedures of Electrochemotherapy) study in 2006, concluded that IV or IT Bleomycin were comparable when given to tumors of volumes less than 0.5 cm³⁵². For Cisplatin, the studies have shown that IT is more effective than the IV route, with CR rates of 82% vs. 48% respectively^{19, 45}. In the ESOPE study local tumor control was achieved in up to 88% of tumors treated with IV Bleomycin, 73% with IT Bleomycin and 75% with IT Cisplatin⁵².

If a lack of uniformity of drug distribution within the tumor is anticipated either by the presence of large and extensive disease, harder fibrotic tumor nodules, lymphedema or limb fibrosis, the IV route would be more suitable⁵². The Intratumoral route is more feasible for those less perfused nodules located in previously pretreated areas¹⁹.

When Bleomycin is given IV the dose is 15,000 IU/m² of body surface area in a bolus lasting 30-45 seconds. But when the IT route is chosen, both drugs are given in a dose calculation based on tumor burden. The IT dose of Bleomycin based on tumor size is calculated as follows: for tumor nodules less than 0.5 cm³ a dose of 1000 IU/cm³, tumor nodules between 0.5 and 1 cm³ a dose of 500 IU/cm³ and for tumors larger than 1 cm³ a dose of 250IU/cm³ is

administered. The cumulative dose should not exceed 400,000 IU/m² due to the cumulative risk of lung fibrosis^{46,52}. If the cumulative dose surpasses 60,000 IU /m², objective respiratory function tests should be made at intervals and the drug discontinued if the respiratory diffusion capacity is abnormal⁶⁰. The IT dose of Cisplatin is given as follow: for nodules less than 0.5 cm³ a dose of 2mg/cm³, for nodules between 0.5-1 cm³, a dose of 1mg/cm³ and for nodules larger than 1 cm³ a dose of 0.5mg/cm³ is given. (Table 1)

Tumor Volume ($V=ab^2\pi/6$)	Bleomycin dose (1000 IU/mL concentration)	Cisplatin dose (2mg/mL concentration)
<0.5 cm ³	1 mL/cm ³ tumor 0.5 mL/cm ³ tumor 0.25 mL/cm ³ tumor	
>0.5 < 1 cm ³		
>1 cm ³		

Table 1. Tumor volume and IT drug dose concentration

The rationale for reducing the intralesional dose per cm³ when treating areas with larger tumor burdens is to reduce the risk of systemic toxicities of the absorbed drug without compromising local efficacy¹⁹.

Prophylactic antibiotics against skin flora should be given intravenously prior to the start of the procedure, especially when lesions are ulcerated or necrotic.

The required time for the procedure is short, with a median treatment duration of 25 minutes³². Repeated treatments are usually well tolerated by the patients^{11, 65-66} and these can usually be performed at 1-6 weekly intervals, without evident resistance⁴⁵.

5. Patient monitoring

Prior to each treatment session patients should have an electrocardiogram (ECG), blood work for evaluation of renal function, coagulation and electrolyte values. All lesions should be photographed and the tumor burden documented. Cellular debris may be released from the tumour during electroporation and these may interfere with the clearance of cytotoxic drugs by the kidney. Thus, when using IV Bleomycin, the serum creatinine should be maintained at less than 150mol/L to ensure proper renal clearance. Physiological monitoring includes visual display of O₂ saturation, pulse rate, blood pressure, continuous ECG tracing and respiratory parameters. Acetaminophen or an anti-histaminic medication may be given to prevent the mild febrile reaction that may occur in the early post procedural period when Bleomycin is administered^{46, 59}.

Technical pearls

1. Test the device and the electrodes prior to beginning the procedure
2. Prep and drape following sterile technique principles
3. Keep in mind correct timing of drug administration and application of the electric current
4. Protect osseous surfaces by tenting the skin
5. Communicate with awake patients and assisting staff, prior to delivery of electric current
6. Check electroporator traces and waves to confirm proper current delivery
7. Do not overlap treating fields with normal tissue
8. Dress wounds according to presenting symptoms

5. ECT development and clinical applications in melanoma

Neumann in 1982⁶⁷ published the first paper regarding EP as a method to transfer genes into mammalian and bacterial cells. There were other *in vitro*^{65, 68} and *in vivo* studies in early and late 1980's using EP in combination with drugs⁶⁹, but the first clinical trials demonstrating its effectiveness over Bleomycin alone for the treatment of a diversity of cutaneous tumors of the head and neck region, were published in early 90's by Mir et al⁴⁷ and Belehradec et al.⁷⁰ from the Institute Gustave Roussy, in Villejuif France. These first 8 patients were treated for squamous cell carcinoma tumors and a complete response (CR) was observed in 57% of the lesions. These early publications encouraged other investigators to expand the principles of this technique to other tumor types including basal cell carcinomas, Kaposi sarcoma, and melanoma metastases^{6, 62, 71-74}. By the mid 90's, Rudolf et al.⁷¹ and Heller et al.⁷³ published the results of an initial small group (5) of melanoma patients that underwent treatment with ECT and IV Bleomycin. They reported overall response rates (OR) in 92% and 50% of 24 and 10 metastatic nodules respectively¹⁹. That same year, Glass et al.⁷⁵ from the University of South Florida in Tampa, USA, reported on the first study using ECT with IT Bleomycin in melanoma metastases obtaining OR rates of 92% (78% CR and 14 % PR) in 20 metastatic lesion – results similar to those obtained by the IV route in the previous studies. Later in 1998, the same group of investigators published the results of a bigger cohort of patients with a variety of cutaneous and subcutaneous malignant nodules. Twelve of 34 patients had 84 metastatic melanoma nodules with documented OR rates up to 99% (89% complete response (CR) and 10 % partial response (PR))⁷⁶. That same year, the combined data produced by five institutions in USA, France and Slovenia was published by Mir et al.³⁹ In this study, twenty patients with metastatic melanoma lesions showed responses in 131 (92%) of 142 lesions, with CR of 53% and PR of 39%. A major finding of this study was that the results were comparable among the institutions even though their treatment protocols and the route used for administering the Bleomycin were not standardized. Additional small studies^{56, 77-79} using ECT with IT Bleomycin for melanoma lesions continued to show good responses, with OR rates of 71 to 100% (CR 23%-100% and PR 0%-62%). Rols et al.⁸⁰ in 2000 continued to demonstrate OR's of 93% using ECT and IV Bleomycin in the treatment of 54 metastatic melanoma nodules.

Sersa et al.⁸¹⁻⁸² introduced Cisplatin as a therapeutic option in 1998. In their studies they reported CR rates of 100% for 2 patients with 13 lesions treated with IT Cisplatin, but low CR rates of 11 % in 9 patients with 27 lesions treated using the IV route. Additional studies were published using this drug by the IT route only^{61, 82-83} with OR rates ranging from 81% to 100% (CR 0%-70 % and PR 6%-100%).

The main issue with most of the studies of the 1990's and early 2000's was the utilization of a variety of treatment protocols with different pulse parameters and pulse generators in combination with different electrode types and drug administration routes^{6, 39, 44, 47, 52, 56, 58, 63, 70, 72-73, 75-78, 81-88}. But in 2006 the results of a pivotal prospective non-randomized multicenter ESOPE study⁵² were published, thus providing recommendations for a standardized protocol for the procedure. The study included 102 patients, 61 evaluated for response and 41 for toxicity respectively. The protocol allowed for administration of Bleomycin either IV or IT, or with Cisplatin IT, and included several histological types of lesions. Ninety-eight lesions from 20 melanoma patients were evaluated - The OR rates were 81% with a CR of 66%. The results confirmed the effectiveness of ECT in the treatment of lesions of different histology, demonstrating an 85% objective response rate and a CR rate of 74% for all lesions.

These results were independent of the drug used or the route of administration chosen. Additionally subsequent studies ^{11, 49, 66, 89-92} of ECT evaluating its effect in the treatment of melanoma and other skin cancers continue to demonstrate the efficacy of the treatment, with response rates comparable to the earlier studies, ranging from 46-100%.

Repeated treatments are feasible as demonstrated by Campana et al.⁹⁰ and Quaglino et al.¹¹ producing additional clinical responses in patients who had initial non or partial responses or who presented with new recurrent lesions. In the Campana⁹⁰ study 34 patients out of 52 were diagnosed with unresectable melanoma, but the response rate for the entire cohort of patients treated with either IT or IV Bleomycin improved significantly from a CR of 50%, up to 83 % after the third ECT treatments.

There are approximately 60 institutions in Europe and in the United States that continue to investigate and offer ECT as a palliative treatment for a variety of unresectable tumors, including melanoma; and in an occasional report it has been used as an alternative curative therapy ⁹³.

6. ECT in the treatment of advanced melanoma

In patients with unresectable recurrent or in-transit melanoma disease who are not candidates for standard surgical or medical treatment, ECT is now an important therapeutic option^{18,43,49,75,84,94}. These cases include those with unresectable disease due to the extensive number of nodules or lesions located in compromising anatomic areas, such as those around joints, nerves, distal leg and in previously operated fields. Encouraging results with long term remissions have been documented ^{17, 45, 78, 93, 95-96}. (Figure 3)



Fig. 3. A) An Exophytic type recurrent malignant melanoma after isolated limb perfusion. B) Six months following treatment by ECT .

Author	No. Patients	Nodules	Drug/route	CRR %	ORR (PR+CR)%
Rudolf 1995 ¹³⁵	2	24	Bleomycin IV	92	92
Heller 1996 ¹³⁷	3	10	Bleomycin IV	30	50
Glass 1996 ¹⁴⁰	5	23	Bleomycin IT	78	96
Heller 1998 ¹⁴¹	12	84	Bleomycin IT	75	99
Kubota 1998 ¹⁴⁴	1	8	Bleomycin IT	100	100
Mir 1998 ²⁷	20	142	Bleomycin IV	53	92
Sersa 1998 ¹⁴⁶	2	13	Cisplatin IT	100	100
Sersa 2000 ¹⁴⁷	9	27	Cisplatin IV	11	48
Sersa 2000 ¹⁵³	10	82	Cisplatin IT	80	87
Rols 2000 ¹⁴⁵	4	55	Bleomycin IV	9	93
Gehl 2000 ¹²⁸	1	9	Bleomycin IT	100	100
Rodriguez 2001 ¹⁴³	2	13	Bleomycin IT	23	85
Sersa 2003 ¹⁴⁹	14	211	Cisplatin IT	70	81
Byrne 2005 ¹⁴⁴	21	52	Bleomycin IT	63	71
Snoj 2005 ¹⁴⁸	1	1	Cisplatin IT	0	100
Marty 2006 ¹²⁴	20	98	Bleomycin IT/IV* larger 3 cm	66	81
Gaudy 2006 ¹⁵⁹	12 (7 per protocol)	30	Bleomycin IT	36	46
		23		74	82
Snoj 2006 ¹⁶⁵	1	16	Cisplatin IT	100	100
Larkin 2007 ¹²¹	4 (2 pts LTF)	56	Bleomycin IT or IV for nodules > 3 cm	0	50
Snoj 2007 ¹⁶⁴	1 (RETC x4)	224	Bleomycin IV	100	100
Tauceri 2007 ¹⁵⁷	3	ns	Bleomycin IT	ns	ns
Qualigno 2008 ¹²	14 (7 pts RETC)	160	Bleomycin IV	50	93
		73		58	93
Campana 2009 ¹⁵⁸	34 MM *(52 cohort 27 RETC)	373	Bleomycin IT/IV	ns	ns
		608		50	74
		257		66	90

IV: Intravenous, IT: Intratumoral, CRR: Complete Response Rate, ORR: Overall Response Rate, PR: Partial Response, CR: Complete Response

RETC: Repeated Electrochemotherapy, LTF: Lost to follow up, ns: No specified, MM: Malignant melanoma

*: Patients with different histological types

Table 2. Summary of most relevant studies using ECT for unresectable or In-Transit Melanoma

Its effectiveness has been proven when providing palliative treatment for hemorrhagic and painful tumor nodules^{56-57, 79, 97-98}. This is a benefit believed to be secondary to the “vascular lock” phenomenon. The vasoconstriction at the arteriolar level produces an immediate and dramatic reduction of perfusion of the malignant lesions, thus controlling the bleeding^{18, 45, 98}. ECT can also be useful as a neoadjuvant treatment for cytoreduction and organ sparing treatment. Its benefits has been reported in patients with perineal melanoma treated with Cisplatin⁸⁵ and for a sphincter saving procedure in anal melanoma⁸³.

ECT can also be suitable for and more tolerable by those patients with a prohibitive surgical risk due to significant comorbidities⁵² because the length of the procedure is relatively short⁵² and patients are able to tolerate multiple sessions. In some anatomical locations ECT could provide good and sometimes better cosmetic results than surgery⁹³.

ECT in combination with cytokine therapy or gene coding immunotherapies has advanced to clinical trials in advanced melanoma. In a phase II study, patients treated with injections of low dose perilesional IL-2 and ECT with Bleomycin, the cytotoxic T lymphocytes response against the known melanoma antigens initially decreased after treatment to reappear when IL-2 was stopped. The tumor-specific peripheral T cells could be detected later in the lesions. The authors theorized that cell death produced by ECT may have attracted and primed dendritic cells with the tumor antigens, which later migrated to the draining lymph node basin, and elicited a T cell response against those antigens expressed by the melanoma¹⁰⁰.

The first Human phase I trial of in vivo DNA electroporation of recurrent malignant melanoma was published in the USA in 2009¹⁰¹. EP with dose escalation of the interleukin-12 plasmid (in vivo DNA EP) was used in the treatment of 24 patients with stage III B/C or IV disease. It resulted in significant necrosis of melanoma cells and regression of the majority of treated lesions. In addition, clinical regression of untreated lesions suggested the induction of systemic anti tumor immune responses. The treatment was found to be safe with no significant reported toxicities. While additional studies in larger cohorts of patients are still necessary, to obtain reproducibility of these results, these data show a new method of inducing potent tumour specific immune responses individual to the patient.

There are no studies comparing ECT with other surgical modalities, however when taking into consideration the learning curve of other complex regional treatment modalities, the techniques of ECT are considered to be “user friendly” and easy to teach and learn. This technique can also be highly advantageous and useful in countries or hospitals where other modalities or resources are limited.⁴⁵

7. Advantages of ECT

1. Excellent local tumor control rates (80-90%)
2. Minimal risk of damage to healthy surrounding tissue
3. Lower chemotherapy doses needed, minimizing the toxicity profile of the drug
4. There is no protein denaturation, which may elicit an undesirable immune response against self antigens
5. High safety profile without severe side effects
6. Good cost/benefit ratio profile: ambulatory setting, lower cost for drugs, and minimal equipment needed.
7. Treatments are well tolerated by patients
8. Improvement in the perceived quality of life

8. Patient selection and limitations of the procedure

The contraindications of the ECT could be divided into drug and procedure related.

A. Drug related contraindications⁶⁰:

1. Known allergy to the drug to be administered
2. Interstitial lung fibrosis, if Bleomycin is going to be used
3. Kidney failure or limited renal function
4. When the cumulative dose of Bleomycin has reached $>400,000$ UI/m²

B. Procedure related contraindications⁴³:

1. For safety reasons ECT should not be used in patients with implanted electric devices such as pacemakers
2. In patients who may carry a higher risk of bleeding such as those on anticoagulants or with increased INR and platelets count $< 70,000$.

9. Limitations

With the current available electrodes, ECT has limitations when treating deep seated tumors⁴³. Tumors larger than 3 cm² appear to have lower response rates (CR 73 %) to ECT^{11,49,90} as compared to nodules smaller than 1cm² (CR 98%). These findings were not affected by either the cutaneous or subcutaneous location of the nodules¹¹.

When tumor nodules are located in irradiated or fibrotic tissues the needle electrode penetration may be problematic with a suboptimal delivery of the electrical current or drugs⁹⁰. Nonetheless, if optimal needle penetration is achieved, ECT is equally effective in irradiated as in non-irradiated tissue⁵². If extensive disease (more than 15 lesions) is present repeated sessions may be necessary. In aggressive disease – while undergoing ECT new cutaneous nodules may emerge but palliative retreatment is worthwhile eventhough the systemic disease progresses rapidly⁶⁰.

This treatment modality has not been studied in randomized trials with other treatment techniques, such as ablative and perfusion or infusion procedures, or radiation therapy. More studies with longer follow up are still needed to evaluate disease free survival and to compare ECT to surgical excision, not only in the palliative setting but as a curative alternative in those patients unsuitable or unfit for a surgical procedure¹⁹.

10. Toxicity and side effects

ECT has a low toxicity profile with limited side effects as compared to other regional therapies such as HILP or ILP⁴¹. However one of the limitations of fully assessing the toxicities of the treatment is the inconsistency of the large majority of the published studies documenting complications.

The systemic dose of Bleomycin is one twentieth of that used in the majority of chemotherapeutic regimens and thus the systemic side effects appear to be limited to nausea^{18,90}. There has been two reported cases of post procedure lypothymia⁹⁰.

The most common local side effects reported by the majority of patients are pain (75%) and erythema limited to the tumor and surrounding treated tissue^{11, 45, 52, 66}. Most of the patients considered those symptoms tolerable as documented by the ESOPE study⁵². The

erythematous reaction usually recedes within a few days¹¹. Local tumor necrosis has been reported in 42% of cases⁶⁶. Delayed wound healing that may take several weeks or months to resolve, and epidermal erosions, and hematomas have been reported as rare events^{66,73,75}. The injection site reactions appear to be low (Type I and II) in the Wiebendirk toxicity scale⁹⁰.

Transient muscle spasms myoclonus, secondary to muscle stimulation by the electrical pulses, have been reported in 25% with lower intensity contractions in up to 78% of patients^{52,66}. Some authors advocate the administration of diazepam to alleviate these particular symptoms^{73,75}. Interestingly the majority of patients are willing to continue treatment if indicated, since the side effects are tolerable¹⁹.

11. What the future holds

Equipment evolution

Bioengineering developments and evolution of the technique continue to expand the applications of ECT as an alternative treatment for tumors that are inaccessible to current electrical probes. A redeveloped electroporator generator provides more flexibility to deliver the electric current at different phases of the cardiac cycle and the facility to connect several electrode probes around the tumor to deliver the electric pulses in synchrony. The other development is related to the type of electrodes. Longer array electrodes are now available to treat larger and deeper seated tumors, which in the past was a limitation of the procedure. These longer electrodes are insulated proximally to prevent short circuiting of current and to protect the normal tissues that are transgressed en-route to the tumor. These new devices have recently been applied clinically^{19, 102-103}. Kos et al. ¹⁰³ have proposed an algorithmic computer optimized analysis to treat deeper tumors, to minimize errors and to maximize treatment benefits.

Another novelty is the creation of finger applicators which allows the application of electrodes into lesions located in difficult to reach anatomic locations, such as the inside of the oral cavity. These finger electrodes have been already tested in the treatment of melanoma of the oral mucosa, and head and neck regions, with complete tumor regression ⁹⁰.

New endoluminal electrodes to reach internal lesions within the gastrointestinal tract have been used in animal models, as well as tumors transplanted into rabbit liver and murine models ^{87, 104-106}. These studies are encouraging and have demonstrated both *in vitro* and *in vivo* (human solid tumor masses in nude mouse models), that the use of flexible electrodes is safe, feasible and reproducible.

There is still the need to create harder needle probes for use in those difficult to treat subcutaneous and cutaneous tumors. These needles must be capable of penetrating into hard fibrotic tissue, while minimizing the bleeding risks and maintaining an adequate electrical distance between the probes.

Nanopulses

Higher amplitude electric pulses or “nanopulses” are currently being evaluated. The use of shorter pulse durations in the nanosecond range are believed to create smaller pores that allow ions but not large molecules to penetrate the membrane. These higher electric fields increase the possibility of producing non resealable pores, thus producing the effect of irreversible electroporation, and consequently allowing the cells to lose their cytoplasm with concomitant cell death. This principle is being used for tumor ablation, palliation or both ⁴³.

¹⁰⁷⁻¹⁰⁹. However in the treatment of melanoma, the advantages of these higher electric fields are not immediately evident.

ECT combination with Gene transfer, immunology and nanomolecules

Several studies suggest that the immune system is also involved in the mechanisms of response to ECT treatment and that this could be exploited for systemic disease control.

In a murine model ¹¹⁴, ECT followed by CpG oligonucleotide injection locally, produced an enhancement of the complete regression responses of tumors from 43 to 100 % , while also triggering systemic antitumor phenomenon with specific immune memory. Activation of dendritic cells released from the tumors are believed to be involved in this response with a recruitment of CD11c and CD11b receptors and an increase of TLR9 expression .

EP in combination with gene transfer is termed "Gene Electrotransfer ". It uses gene coding plasmids in order to transfer intracellularly a combination of genes, to either knock down the expression of a particular gene, or to stimulate temporary patterns of gene expression). The technique allows for avoidance of the biohazard issues intrinsic to viral vectors ^{101, 115-116}. Scientists at the Cork Cancer Research Center in Ireland have investigated in an *in vivo* murine model, the application of EP and local gene therapy for malignant tumors. A plasmid coding for two immunogenes, granulocyte-macrophage colony-stimulating factor (GM-CSF) and the B7-1 co-stimulatory immune molecule were delivered by EP. This resulted in the complete regression of the majority (60%) of non immunogenic tumors, while eliciting a tumour specific systemic response that hindered the metastatic growth in the liver in a 100 % of the mice. This was a durable potent response with tumor specificity. When the remaining non responders non responders tumors were excised, an improved survival was observed when compared to the control groups, thus suggesting that the use of neoadjuvant electroporation and gene therapy given at an appropriate time interval prior to tumour excision or ablation could prevent the surfacing of metastatic disease ¹¹⁷⁻¹¹⁹. Regulatory T cell depletion at the time of electrogenetherapy stimulated improved complete response rates from 60 to near 100% suggesting a potential for improving efficacy of electroporation based immunogene therapy.

ECT with injection of TNF- α intra or peritumorally and suboptimal doses of Bleomycin in mice might have a positive immunomodulatory effect, and possibly adds a systemic component to the localized ECT treatment¹²⁰ . It has been noted that EP with INF- α used to treat Mycosis Fungoides (subcutaneous lymphoma) lesions produced a 100 % CR ¹²¹ The cytotoxic action of INF- α was attributed to its increased tumoral concentration and the prolonged time of action produced by the EP. ECT either with INF- α or TNF- α might permit the usage of less toxic doses, while enhancing clinical local and systemic immunological response rates and minimizing systemic side effects¹⁹.

ECT, radiotherapy and activation of bioreductive drugs

The "vascular lock" principle of EP has the potential to be used to "activate" bioreductive drugs such as Tirapazamine against neoplastic cells ⁴³, due to its vascular disrupting effect of tumor blood supply. In addition ECT has been shown to have a synergistic effect with radiotherapy in preclinical investigations, opening the possibility to use it as a radiosensitising tool for the palliation of subcutaneous lesion^{43, 122}.

The continued development of improved diagnostic methods will allow for earlier diagnosis of metastases, and possibly open the opportunities for *in situ* neoadjuvant treatment of

higher risk malignant melanomas by means of immunogene or cytokine therapies, hence establishing tumour specific responses which could prevent recurrence and eradicate disseminated micrometastases.

12. Summary

ECT with Bleomycin or Cisplatin is an effective treatment in the palliative management of unresectable recurrent cutaneous or subcutaneous melanoma metastases or in-transit disease, with OR rates of approximately 80-90%. ECT should now be considered as part of the armamentarium for treatment of loco regional advanced melanoma. The technology of ECT continues to evolve allowing for the treatment of metastatic lesions in other organs or anatomic regions. The principles of EP are already being applied in the clinical setting for the delivery of targeted therapies such as gene transfer and immunotherapy. These therapies along with ECT have the potential not only for local, but for distant treatment of tumors such as those of malignant melanoma, by stimulating a self-driven immune response to achieve systemic control of the disease

However studies evaluating long term follow up results of ECT in melanoma are still needed prior to considering it as an option for curative intent. ECT has been proven to be an excellent palliative option in treatment of recurrent unresectable or in-transit disease.

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Pulse Power Ablation of Melanoma with Nanosecond Pulsed Electric Fields

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1. Introduction

Melanocytes are cells that originate in the neural crest. In the dermis, their well characterized role is to produce melanin and through interactions with keratinocytes transfer this pigment to determine skin color and protect the largest organ in the body, the skin, from ultraviolet light. As an effective free radical scavenger, melanin protects against reactive oxygen species that would otherwise damage DNA [Rózanowska et al., 1999]. Melanocytes may also have other roles such as immune, neuroendocrine, and signaling functions through interactions with cells other than keratinocytes, such as lymphocytes, mast cells and endothelial cells [see Tsatmali et al., 2002 for review]. However, keratinocytes regulate melanocyte number, differentiation and melanin production in response to UV radiation. It may be that the resilience of melanocytes to protect the skin, their extraordinary regenerative capacity and their origin as neural crest migratory cells makes them one of the most deadly forms of metastatic skin cancers when they undergo tumorigenesis. It is known that there is a great deal of common cellular and genetic events among embryonic development, tissue regeneration and cancer. Further, typical self-renewal and migration capacity of cancer are shared with embryonic and regenerative cells [White and Zon, 2008]. The recapitulation of embryonic genetic programs is facilitated by overexposure to extreme sunlight (UVA and UVB) or tanning bed light (mostly UVA). The American Cancer Society estimated that in the US in 2009, 68,720 new cases of melanoma (188 new cases /day) will be diagnosed and 8,650 people will die from the disease (24 melanoma deaths /day). The continued increase in melanoma is a significant cause of morbidity and mortality in the Western world. Thus, metastatic melanoma remains a persistent therapeutic challenge. There are limited successes in preventing this often fatal disease and there are even fewer successes in developing a cure.

2. Standard melanoma therapies

Presently available treatment strategies have had limited impact on progression and overall survival of patients with melanoma. In addition to surgery, currently approved treatments for metastatic melanoma include chemotherapy, radiation and/or immunotherapy. Chemotherapy is mostly limited to dacarbazine, cisplatin, carmustine and vinblastin. The addition of systemic immunotherapy with IL-2 and/or INF α to these chemotherapeutic

agents resulted in major toxicities. INF α is approved for use but is not included as a standard of care due to minimal impact on overall survival and significant toxicities [Amaravadi et al., 2007; Gogas et al., 2007; Tarhini et al., 2006]. Unfortunately, metastatic melanoma is one of the most resistant cancers to a wide range of treatment modalities including single-agents and combination chemotherapy, immunotherapy, chemoimmunotherapy and a host of immune stimulators [Riker et al., 2007].

3. Targeted melanoma therapies

One of the major problems that cancer therapeutics face today is coping with a diversity of cancer diseases and melanoma is no exception. However, Hanahan and Weinberg [2000, 2011] reasoned that since all mammalian cells express the same molecular mechanisms for proliferation, differentiation and death, most if not all cancers should share a limited number of common molecular, biochemical and cellular traits that govern their behavior. This is insightful since cancers exhibit hundreds of genotypes and subtypes of tumors can be found in specific organs. In addition, different mutations can be found within the same tumor. In order to promote the development of cancer research into a more logical science, to provide more focused characterization of cancer and to manage this array of diseases, Hanahan and Weinberg [2000,2011] initially defined six major hallmarks of cancer that exhibit physiological anomalies that control cell homeostasis and proliferation. These include self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. They later included reprogramming of energy metabolism and evasion of immune detection as cancer hallmarks [2011]. Kroemer and Pouyssegur [2007] included evasion of immune surveillance as a seventh hallmark. Luo et al. [7] expanded these classic hallmarks to include stress phenotypes of tumorigenesis and defined a large class of non-oncogenes that are essential for cancer cell survival. Treatment strategies to develop targeted, mutation-specific small molecule drugs or monoclonal antibodies to treat melanomas is an outcome of an evolving understanding of the molecular mechanisms of melanoma in the perspective of these defined cancer hallmarks.

A number of new agents for advanced melanoma are being tested in clinical trials. Considering the hallmarks of cancer, the most recent new drugs for treating melanoma have found some successes in the inhibition of self sufficiency of growth signals, immune surveillance evasion, sustained angiogenesis and evasion of apoptosis. These agents are directed towards proteins that are involved in cell signaling pathways that are responsible for cell division and proliferation, immune responses, blood vessel formation and programmed cell death. Several considerations are appropriate when taking targeted drugs into account. First, targeted therapies are generally effective for individuals identified with a specific mutation, so patients can be screened before treatment to determine if they have the specific mutation for which the drug is designed to affect. This exemplifies the impact of personalized medicine on oncology and if successful will become a common procedure. This can be achieved by relatively simple and inexpensive procedures. The second concern is issues of heterogeneity within a patient's melanoma. While a specific mutation may be determined in a biopsy sample, the targeted mutation may not be present in all of the patient's melanoma cells. This provides a potential means for resistance and recurrences. A third issue is the continued "pressure" exerted or relieved by a targeted agent on cell signaling in the affected cancer cells. Such events modify signaling dynamics with responses

that attempt to “escape” the modification, which may also lead to resistance and recurrences. These issues may complicate uses of targeted medicine in melanoma treatments.

Although other causes of cancer are known, mutations that often lead to malignant growth and metastasis frequently occur in protein kinases [Blume-Jensen and Hunter, 2001; Chenga and Force, 2010]. Protein tyrosine kinases transduce extracellular signals into intracellular functions that regulate a wide array of cellular activity. Tumorigenesis is often driven by constitutively active protein kinases that modulate cell cycle, angiogenesis or apoptosis. Two general classes of protein kinase inhibitors include monoclonal antibodies or small molecular weight inhibitors that are directed against specific protein tyrosine kinase receptors or their ligands. A common site for protein kinase inhibitor drug design is the ATP binding site because transfer of phosphate to substrates cannot occur without the gamma-phosphate from ATP. However, the binding sites are so highly conserved among kinases that kinase genes can be identified without phosphotransferase activity by the presence of specific sequences that define ATP binding. Two major concerns in designing these drugs are issues of specificity and potency. Specificity is one issue because of the ATP binding site conservation among all protein kinases. Given the broad range of kinase functions, it becomes important to inhibit kinases involved in tumorigenesis without affecting other kinases. Potency is the other concern because ATP is at relative high concentrations in the cell (milliMolar) and an orally active small molecular weight drug must have a high affinity to be an effective inhibitor. Kinase inhibitors are divided into three major classes based on binding to their ATP site [Chenga and Force, 2010]. Type I inhibitors have high affinity for the ATP binding pocket. Therefore type I kinase inhibitors generally have broad kinase specificity. Type II kinase inhibitor (e.g., imatinib) are more selective because they not only have specificity for the ATP binding site but also sites adjacent the ATP binding site. In addition, unlike type I inhibitors, type II inhibitors can bind to kinases in their inactive conformation. Type III kinase inhibitors (e.g., families of mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitors) are more selective but more difficult to design because they bind to sites distant from the ATP binding pocket.

A number of monoclonal antibodies and targeted small molecular weight protein tyrosine kinase inhibitors are in clinical trials for the treatment of melanoma. Regarding the hallmarks of cancer, these agents are directed to prevent limitless replicative potential and/or self-sufficiency in growth signals, evasion of immune surveillance, sustained angiogenesis, and evasion of apoptosis. Because protein tyrosine kinases are involved in many of these hallmarks, many of these agents are multi-protein tyrosine kinase inhibitors that are directed towards more than one of these hallmarks and kinases so have overlapping targets. For example, Sunitinib, Sorafenib and Imatinib target VEGFRs and/or PDGFRs and can act as anti-angiogenesis agents. In addition, these drugs can also act on either c-Kit and/or Raf-1/B-Raf and are directed towards limitless replicative potential and/or self-sufficiency in growth signals. Current data indicate that melanomas with activating c-KIT mutations and possibly also with KIT gene amplifications respond to therapy with tyrosine kinase inhibitors blocking c-KIT. It was suggested that subgroups of patients with metastatic melanoma prone to *KIT* mutations, such as primary mucosal and acrolentiginous melanomas, should be analyzed for their *KIT* status [Satzger et al., 2010].

A significant number of melanomas are mutated at V600E in BRAF (~60%), an oncogene that is a signal transduction enzyme near the origin or proximal end of a cascade of phosphorylation events that promotes proliferation through the Raf/Mek/Erk pathway and

survival of cells through the PI3K/Akt/mTor pathway. PLX4032, a B-raf kinase inhibitor has been shown to induce programmed cell death by inhibiting proliferation, growth and survival. It is also known to be involved in overcoming apoptosis evasion. In a recent multi-phase phase I dose-escalation trial among 16 patients with melanoma exhibiting the V600E BRAF mutation, 62% (10 patients) had a partial response and 1 had a complete response when given PLX4032 (240mg or more twice daily). Among 32 patients in the extension cohort receiving as much as 960mg twice daily, 75% (24 patients) had a partial response and 2 patients had a complete response. The estimated median progression-free survival among all patients was more than 7 months [Flaherty et al., 2010]. This represents an important new therapeutic development in the treatment of melanoma. For those who experienced relapses, second mutation(s) continue to drive tumorigenesis. Two mechanism of resistance to PLX4032 (covering 40% of cases) have been discovered. In one of these the cancer cells begin to overexpress a cell surface Beta-type protein platelet derived growth factor receptor creating an alternate survival pathway. In a second mechanism of resistance, the oncogene *N-Ras* mutates, reactivating the normal BRAF survival pathway [Nazarian et al., 2010].

Several new agents have been evaluated for targeting the immune system. Several agonist monoclonal antibodies have shown promise including those directed against members of the tumor necrosis factor receptor superfamily such as 4-1BB (CD137), Ox40 (CD134), and CD40. Two human antagonists monoclonal antibodies have been investigated in melanoma patients that bind to CTLA-4 (cytotoxic T lymphocyte-associated antigen 4), which is a molecule on helper T-cells that appears to play an important role in regulating natural immune responses. These include Ipilimumab and Tremelimumab. Ipilimumab was recently evaluated alone and in combination with gp100 as compared with gp100 alone. In this phase III trial, it showed improved overall survival in patients with previously treated metastatic melanoma. Although there were some severe adverse reactions associated with this therapy they were reversible with appropriate treatment [Hodi et al., 2010; Peggs and Quezada, 2010]. Tremelimumab has been shown to induce durable tumor responses in patients with metastatic melanoma in Phase I and Phase II clinical studies [Reubens et al., 2006] and more recently has shown promise in treatment of patients with metastatic melanoma, in a completed randomized, double-blind phase III trial [Callahan et al., 2010].

While most of the potential therapeutic agents are given orally, other delivery methods have begun to be used for more localized treatments and include the utilization of electric fields. One of these approaches is to maintain cell survival using conventional electroporation to deliver plasmids that express genes for therapeutic effects [Neumann et al., 2002]. This method is referred to as electrogene therapy. This is an outgrowth of electrochemotherapy that uses conventional electroporation to deliver impermeable chemotherapeutic drugs such as bleomycin to tumors [Heller et al., 1996; Mir et al., 1991]. The delivery of IL-12 to melanoma lesions to activate the immune system against melanoma by electrogene therapy has demonstrated safety and efficacy in phase I clinical trials. Results demonstrated a bystander effect where lesions surrounding electrogene-treated lesions showed tumor regression [Daud et al., 2008]. The use of electrogene therapy to deliver plasmids for gene expression in pre-clinical and clinical trials is reviewed elsewhere [Beebe et al., 2010].

Two other therapies are based on uses of electric fields that extend conventional electroporation (EP). The first is irreversible electroporation (IRE), which extends EP by increasing the electric field to produce cell necrosis through irreversible plasma cell membrane defects [Al-Sakere et al., 2007; Davalos et al., 2005; Onik et al., 2007;], although some evidence suggests that ablation zones may also exhibit apoptotic cells [Guo et al., 2010;

Lee et al., 2010], a possibility that requires further investigation. IRE is also reported to affect only cell membranes and limits effects to cells within the ablation zone, sparing blood vessels, bile ducts and extracellular matrix structures [Maor et al., 2007; Phillips et al., 2010]. However, a major drawback is muscle contractions induced by microsecond pulses that are absent or significantly reduced with nanosecond pulses in a porcine model [Long et al., 2011]. While IRE could be considered a blunt tool that forces cell death by necrosis (or apoptosis), it exhibits precision for applications because it can clearly define the intended treatment zone and spares larger vessels and ducts [Ivorra et al., 2009; Granot et al., 2009]. Another extension of conventional electroporation uses pulse power technology with nanosecond pulse electric fields (nsPEFs), which continues to be explored for tumor ablation. While there are some comparisons with conventional electroporation, pulse power is distinct in several regards. Compared to conventional electroporation, pulse power ablation (PPA) with nsPEFs uses exceptionally short pulse durations (ns vs. micro-second or milli-second) with exceptionally fast rise times (2-4ns vs. ~100ns). It is hypothesized that fast rise times with short pulse durations have advantages for intracellular effects [Schoenbach et al., 2001]. The electric fields used in these applications of pulse power are higher than conventional electroporation (10-350 kV/cm vs. about 1 kV/cm). Further, the power deposited in cells or tissues is much higher (~180MW vs. ~500W) and energy is lower (mJ/cc vs. J/cc); they are non-thermal. The combination of these conditions causes cell membrane supra-electroporation in *all* cellular membranes resulting in high density "nanopores" (nm diameter) [Gowrishankar et al., 2006; Stewart et al., 2004,].

4. Applications of ultra-high pulse power ablation for the treatment of melanoma

Pulsed power is a technology designed to store energy and release it very quickly to produce immediate high power. It was initially developed during World War II for use in radar, which requires short high power pulses. Since then pulse power technology has been used in particle accelerators, ultra-strong magnetic fields, electromagnetics, fusion research and high power pulsed lasers. An example of how pulse power works is to compare the storage of one joule of energy released in one second versus releasing the same energy in one microsecond or one nanosecond [Wikipedia]. If the stored joule of energy is released all at once to a suspension of cells or tumor tissue in one second, the peak power delivered would only be 1 watt. If all of the stored energy were released within one microsecond, the power would be one megawatt, a million times greater and if release in one nanosecond the peak power would be one gigawatt, a billion times greater. Within the last ten years, applications of ultra-high pulse power have been extended to biology and medicine [Beebe et al., 2002, 2003a, 2004; Beebe and Schoenbach, 2005; Schoenbach et al., 2001, 2004; Vernier et al., 2003a,b], including the treatment of melanoma [Chen, 2009, 2010; Ford et al., 2010; Nuccitelli et al., 2006, 2009, 2010]. In applications of pulse power ablation for treatment of melanoma, as much as 6 kV of potential energy were release in multiple 300ns bursts at 60kV/cm into murine B16f10 melanoma cells or tumors. In addition, the pulses included an extremely fast rise and fall time of about 4-5ns so the 300ns pulses were at maximum power for about 97% of each pulse. It is hypothesized, but not yet proven, that this rapid rise time is important for targeting intracellular organelles and may be an important aspect for therapeutic efficacy.

Another factor that results from nsPEF conditions is the absence of significant temperature increases during treatment. Both *in vitro* and *in vivo* nsPEF studies have shown non-thermal

effects [Chen et al., 2009; Nuccitelli et al., 2006; Pakhomov et al., 2004;]. However, the presence or absence of thermal effects depends on the pulse repetition rate and heat dissipation rates of *in vitro* and *in vivo* systems. Higher frequency applications are more prone to thermal effects. While initial strategies for biological and medical applications were to achieve non-thermal effects, there may be advantages of synergistic effects between electric fields and heating.

A combination of an ultrashort pulses and rapid rise times (shorter than the charging and relaxation time of plasma membranes), created conditions that were hypothesized and shown to have effects on permeabilization of intracellular granules in calcein-loaded human eosinophils without calcein release through the plasma membrane [Schoenbach et al., 2001]. Selective permeabilization of intracellular vesicles was also shown using a mixed population of phospholipid vesicles as well as in endosomal membrane vacuoles in COS-7 cells [Tekle et al., 2005]. Several other studies demonstrated effects that suggested selective permeabilization of intracellular calcium storage sites [Beebe et al., 2003b, 2004; Buescher and Schoenbach, 2003; Vernier et al., 2003a; White et al., 2004] and effects on plasma membranes that were unique compared to conventional electroporation pulses [Beebe et al., 2003a; Deng et al., 2004; Ibey et al., 2010]. However, it was shown later that apparent absences of plasma membrane effects were likely due to formation of plasma membrane pores that were smaller than the reporter molecules, such as propidium iodide or ethidium homodimer [Bowman et al., 2010; Pakhomov et al., 2007a, b]. While these nsPEF applications are known to have effects on all cell membranes, differences among cell types and their sensitivities to pulse power conditions have been reported and will be discussed here.

NsPEF effects have been observed on plasma membranes and intracellular membranes of the cytoskeletal structure, endoplasmic reticulum, mitochondria and nucleus. Many of these effects are hypothesized to be caused by the formation of "nanopores", which occur during supra-electroporation [Gowrishankar et al., 2006; Stewart et al., 2004]. As stated earlier, this effect includes small pores on a large proportion of all cell membranes as opposed to larger pores on a small proportion of the cells membranes during conventional electroporation. However, this may need to be re-considered since more recent modeling approaches suggest that electroporation pulses generate fields inside cells that are high enough to permeabilize intracellular membranes and vesicles [Esser et al., 2010]. This paper predicts for EP that these membrane pores expand and become larger than nanopores arising from nsPEF. It will be important to experimentally test this in cells and tissues to discern real differences between conventional electroporation and nsPEFs concerning intracellular effects and therapeutic relevance. Nevertheless, nanopores allow small ion transport especially ions like sodium, calcium and potassium that can affect cell excitability. Conventional electroporation pores occur primarily in plasma membranes and allow transport of ions and larger molecules such as impermeable chemotherapeutic drugs and plasmids that express therapeutic genes. Like all dose related biological effects, the induction of nanopores has a threshold related to the pulse characteristics including duration, electric field and number. Thus, as pulse conditions increase to longer pulse durations, nanopores will begin to expand and assume characteristics of conventional electroporation. Thus, as pulse durations increase from very short durations to longer duration, there is a continuum from the presence of nanopores on all cell membranes to larger pores primarily on plasma membranes. Further, each cell type has different thresholds presumably based on cell membrane characteristics.

In addition to pulse duration alone, other pulse conditions such as pulse amplitude and pulse number can also influence cell behavior. Modeling results using molecular dynamics [Hu et al., 2006; Tieleman et al., 2003; Vernier et al., 2006] and continuum models [Joshi et al., 2001; 2002; Kotnic and Miklavcic, 2006; Smith et al., 2006] have provided insight into the mechanism of permeabilization of membranes with a single pulse. From an experimental point, Jurkat cells treated with 10ns and 60ns single pulses at various electric fields demonstrated an enhanced uptake of ethidium homodimer, a marker for membrane integrity, and an enhanced binding of Annexin V, a marker for phosphatidylserine externalization, both indicative of membrane permeabilization [Beebe et al., 2004]. In studies using Jurkat and U937 cells [Pakhomov et al., 2004] or GH3 and CHO cells [Ibey et al., 2009] under a variety of conditions, viability scaled with the absorbed dose as defined as the electrical energy density. Another study evaluating cell viability of murine B16f10 melanoma cells with trypan blue using 0.8ns pulses seemed to confirm this [Schoenbach et al., 2008]. However these studies used different cell types and different pulse condition variables but did not include conditions of variable pulse durations, amplitudes and numbers together. When all three variables were included, the results scales with the product of the pulse duration (τ), pulse amplitude (E) and the square root of the pulse number ($n^{0.5}$). This square root dependence on the pulse number indicates a statistical motion of cells between pulses with respect to the applied electric field, and can be explained using an extension of the random walk statistical results to random rotations of cells in solution [Schoenbach et al., 2009]. These studies can be of significant value to determine the underlying interaction mechanism(s) between pulsed electric fields and cells and tissues. Collectively, these data suggest that there are multiple mechanisms of action of nsPEFs on biological systems.

When effects of nsPEFs on cells are considered, it remains to be determined which effects are due to direct actions of electric fields on cell structure and function and which are due to secondary downstream cellular or biological effects that include responses from the cytoskeleton, endoplasmic reticulum, mitochondria, nucleus and DNA damage. Given conventional electroporation effects on cell membranes and now reports of nsPEF effects on all cell membranes including intracellular structures, it becomes difficult to determine which effects are direct and which are indirect. It seems clear that nanopore formation is a direct electric field effect, but it remains to be seen whether these nanopores are primarily responsible for initiation of all observed nsPEF effects, from specific structural effects to broadly based effects such as cell survival and cell death. It is possible that effects other than nanopore formation are present that may be unrelated to membrane charging events. However, there are presently no available published data to address this issue. While nanopores must have significant effects on cells, other downstream biological effects are still under investigation. Given that pulse power applications for the purposes of this review are for melanoma tumor ablation, we can ask how nanopores or other consequences of electric fields result in effects that we observe as melanoma cells die and melanoma tumors regress. Here we review literature that has led to understanding these effects from a number of cell types and then to recall some literature examples and present some data using nsPEFs on B16f10 melanoma cells and tumors. Given that pulse power using nanosecond pulsed electric fields can eliminate B16f10 tumors and prevent their return, we will focus on nsPEF effects that bear on hallmarks of cancer including reversing evasion of apoptosis and opposing sustained angiogenesis in tumors *in vivo*. However, both of these effects are related to a number of actions on cell membrane and organelles that lead to cell demise.

5. Effects of ultra-high pulse power on cell plasma membranes

Although pulse power influences both plasma membranes and intracellular membranes, responses from the plasma membrane are relatively easy to analyze. It was initially hypothesized that pulse power fields could pass through the plasma membrane with significant effects. Using propidium iodide and ethidium homodimer as conventional probes for membrane permeability, initial studies suggested that our prediction was correct [Beebe et al., 2003; Schoenbach et al., 2001; Vernier et al., 2003]. However by using smaller fluorescent probes and more sensitive analytical methods, it has become clear that nsPEFs produce unique pores or aqueous channels that are distinct from conventional electroporation pores in that they are much smaller and are thus called nanopores. Pakhomov and coworkers have provided significant detail to the behavior of nanopores induced by nsPEFs [Bowman et al., 2010; Pakhomov et al., 2007a, b, 2009] but have only begun to fully characterize them. Pulses with durations of 60ns and electric fields of 12 kV/cm showed long-lasting (minutes) reduction of the cell membrane resistance and a corresponding loss of the plasma membrane potential. The formation of nanopores was demonstrated by patch clamp analysis and verified by non-electrophysiological methods using Tl⁺-sensitive fluorophore and using Tl⁺ uptake as a marker for nanoporation. These nanopores are voltage sensitive, exhibit an inwardly rectifying current that resembles those of nonselective cation ion channels but do not appear to exhibit outward currents. These pore properties are distinct from conventional electro-pores, disappear when they become larger propidium permeable pores and are not blocked by broad-spectrum K⁺ channel inhibitors or Ca²⁺ chelators. Tl⁺ uptake was observed at electric field intensities far below the threshold for propidium iodide uptake and they remained stable for as long as ten minutes. Overall, nsPEF applications to cells *in vitro* provide evidence from models and experimental observations that they open lipid nanopores that create unique aqueous channels for cation-selective transport into cells from the extracellular environment. These plasma membrane nanopores are likely to be responsible, at least in part, for many nsPEF-induced biological effects. Given that there is evidence for effects on intracellular membranes, it is highly likely that pulse power-induced nanopores are present in intracellular membranes and are responsible for at least some of the biological response from cells as will be discussed later.

The plasma membrane separates cells from the external environment, but is much more than an external covering to contain intracellular organelles. This lipid bilayer includes a wide variety of integral membrane proteins that allow both passive and active transport of ions into and out of cells. In addition, the plasma membrane exhibits symmetry in lipid constituents on the inner and outer leaf of the membrane. Normally, phosphatidylserine is on the inner leaflet and phosphatidyl-ethanolamine is on the outer leaflet. During apoptosis, phosphatidylserine is externalized, signaling to other cells in the micro-environment that these cells are undergoing cell death by apoptosis. Under these conditions macrophages will engulf the dying cells by phagocytosis before cells lose their integrity through membrane permeabilization preventing inflammatory responses. However, since nsPEF conditions that lead to cell death will induce nanopore formation, cells may become permeable to membrane integrity markers such as propidium iodide or ethidium homodimer. Whether cell plasma membranes become permeable depends on EP conditions as discussed earlier. In addition, the cell type is also an important consideration as indicated in some of the following examples.

When ten 100ns pulses at 60kV/cm were applied to 3T3 pre-adipocytes and analyzed by flow cytometry, there was no increase in ethidium homodimer or propidium iodide uptake, indicating that the plasma membrane was impermeable to molecules on the order of about 1 nm or larger. Using Annexin-V-FITC as a phosphatidylserine externalization marker, there was a time-dependent increase in Annexin-V binding but no increase in ethidium homodimer uptake 30 minutes after treatment [Beebe et al., 2003a]. Eighteen to twenty-four hours later less than 10% of the cells survived. These cells also exhibited active caspases, suggesting caspase-associated apoptosis.

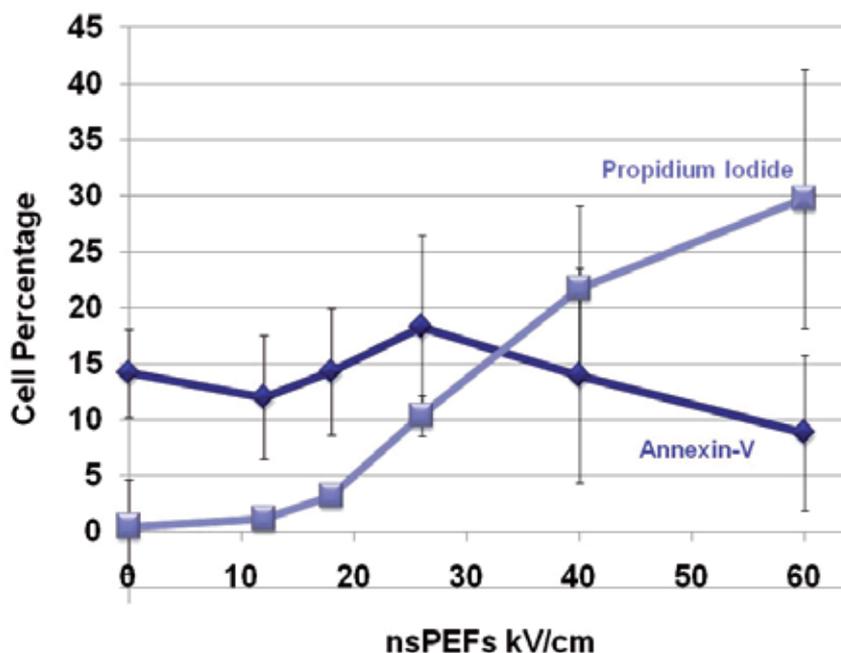


Fig. 1. Effect of nsPEFs on B16f10 melanoma cell permeability and phosphatidylserine orientation

As indicated in Figure 1, B16f10 cells were also exposed to ten 300ns pulses with electric fields as high as 60 kV/cm. In contrast to 3T3 pre-adipocytes under these same conditions, there was very little if any Annexin-V binding indicating no phosphatidylserine externalization. However about 20-40% of cells took up propidium iodide at 60 kV/cm, suggesting that a minority of cells exhibited nanopores larger than about a nanometer. B16f10 cells also exhibited caspase activity (Ford et al., 2010, see below). Thus, 3T3-L1 pre-adipocytes exhibited Annexin-V binding but no propidium iodide uptake while B16f10 cells exhibited no significant Annexin-V binding, but uptake of propidium iodide in a small population of cells. Both cell types showed increases in active caspases. When Jurkat cells were treated with nsPEFs, 300ns pulses at 60 kV/cm resulted in significant and immediate cell necrosis indicating that the plasma membranes of Jurkat cells were significantly more sensitive to pulse power than 3T3-L1 cells. Jurkat cells exposed to 60ns pulses at 60kV/cm exhibited Annexin-V binding but a delayed uptake of propidium [Beebe et al., 2003a]. The delayed permeability to propidium iodide was distinctly different than the immediate

propidium uptake when cells were exposed to 10 microsecond pulses typical of conventional electroporation [Deng et al., 2004]. When HCT116 cells that were wild-type and null for p53 were treated with nsPEFs, addition of ethidium homodimer-1 and Annexin-V-FITC post-pulse demonstrated greater fluorescence in p53 null cells versus p53 wild-type cells, suggesting a p53-dependent biological effect on plasma membranes [Hall et al., 2005]. It is possible that relative levels of p53 may affect plasma membrane response to pulse power.

The comparisons among these cell types demonstrates that different cells have unique responses to the same nsPEF conditions and that some cells respond to lower pulse conditions than others. The conclusion that there are cell-type specific responses to pulse power is consistent with several other studies [Hair et al., 2003; Ibey et al., 2010; Stacey et al., 2003]. While these data indicate the possibility to selectively targeting specific cells in a mixed population, specificity for cancer versus normal cells has not been demonstrated. Nevertheless, it is highly likely that when electric fields are sufficiently high, pulse power is expected to eliminate all cell types, including slowly proliferating cancer stem cells, which are not readily affected by chemotherapeutic agents, and host cells that are collaborating with malignant cells.

While propidium iodide uptake is a well-characterized marker for cell viability and phosphatidylserine externalization is a well characterized marker for apoptosis, both of these indicators and their indications should be reconsidered when cells are exposed to nsPEFs or conventional electroporation. It is well known from conventional electroporation studies that cells can be transiently permeable to relative large molecules and remain viable. The primary aim of conventional electroporation is transient membrane permeability in cells that survive. Transient permeability has also been demonstrated using nanosecond pulsed electric fields in HCT116 cells [Hall et al., 2007b]. It was hypothesized from modeling analysis [Hu et al., 2005, 2006; Vernier et al., 2006a, b] and demonstrated experimentally [Vernier et al., 2006] that exposure to nsPEFs could externalize phosphatidylserine through nanopores that were created in the plasma membrane. These nanopores are distinct from pores created by EP in that they are too small to allow transport of large molecules but do allow transport of ions and externalization of PS. Conventional electroporation induces phospholipid rearrangements in plasma membranes [Haest et al., 1997; Schwarz et al., 1999]. Tekle et al. [2008] demonstrate that phosphatidylserine externalization induced by nsPEFs in the absence of caspase activity, resulted in phagocytic clearance of B cells by mouse macrophages in part by electric field-induced apoptosis mimicry. Thus, analysis of phosphatidylserine externalization as an apoptosis marker in response to nsPEFs should be carefully considered and as always demonstration of apoptosis should include several different markers.

It is also possible that nsPEFs have effects on other structures such as those with roles for transport across membranes or other biological effects. For example, ligand receptor interactions or the structure and function of membrane receptors could be affected by pulse power. Effects of pulse power have not been investigated on caveolae, which have been reported to play roles in endocytosis, lipid trafficking and signal transduction; and on lipid rafts, which have been implicated in cell signaling, membrane fluidity and protein and receptor trafficking [Brown and London, 1998]. Thus, while plasma membrane and putative intracellular membrane nanopores are likely important in biological responses to ultra-high pulse power effects on cells and tissues, other membrane structures and functions may also be modulated by pulse power.

6. Effect of nsPEFs on the endoplasmic reticulum

The endoplasmic reticulum is composed of intracellular membranes with a similar composition to the plasma membrane, forming an intracellular network of tubules and cisternae or “little nets” (from the Latin reticulum). It carries out several specialized functions including translation, folding and transport of proteins, sequestration of calcium, glycogen storage, and responses to stress. Given that nsPEFs were hypothesized to have intracellular effects and these effects were most likely due to membrane charging events, it was reasonable to consider that they would have effects on this extensive intracellular membrane array. Since the endoplasmic reticulum is a storage site for calcium, a number of studies demonstrated effects of nsPEFs on calcium mobilization in the presence and absence of extracellular calcium [Beebe et al., 2003b, 2004; Buescher and Schoenbach, 2003; Vernier et al., 2003; White et al., 2004]. In fact, of all the cellular responses that have been elicited with nsPEFs, calcium mobilization is the one of the most sensitive, occurring at shorter pulse durations and lower electric fields than other measured cell responses. In experiments with HL-60 and Jurkat cells [Beebe et al., 2004; White et al., 2004] using 60ns pulses, calcium release was observed from intracellular storage sites in the absence or presence of extracellular calcium chelators at electric fields as low as 2-4kV/cm. Based on studies that depleted calcium stores in the endoplasmic reticulum, it was determined that calcium was mobilized from this site in these cells. For phosphatidylserine externalization in these same cells, 60ns pulses required electric fields 10-20 times higher (40 kV/cm). Calcium mobilization was also observed in Jurkat cells with ten 30ns pulses at 25kV/cm under conditions that did not allow Na⁺ transport across the plasma membrane [Vernier et al., 2003]. nsPEFs also acted as an agonist to activate platelets to form platelet gels [Zhang et al., 2008]. This effect was electric field dependent and was postulated to result from an increase in calcium mobilization through nanopores in the plasma membranes as well as from intracellular calcium stores, most likely alpha-granules in platelets. A study evaluating calcium responses in chromaffin cells exposed to nanosecond electric pulses suggested a role for L-type calcium channels for calcium entry, but not from intracellular calcium [Vernier et al., 2008]. A more recent study exposing chromaffin cells in the presence and absence of a variety of channel inhibitors concluded that 5ns pulses opened multiple types of voltage-gated calcium channels involving sodium transport across plasma membranes by either non-selective cation channels and/or lipid nanopores [Craviso et al., 2010]. Another study analyzed fluorescent calcium sensitive probes in isolated rat ventricular myocytes exposed to pulses of 4ns and electric fields at 10-80 kV/cm [Wang et al., 2009]. This study demonstrated that these ultrashort pulses triggered action potentials through tetrodotoxin-insensitive, non-selective ion channels that were consistent with the presence of nanopores in the sarcolemma.

nsPEFs also mobilized calcium in B16f10 melanoma cells [Ford et al., 2010]. Calcium is involved in most, if not all cell functions [Berridge et al., 2000]. Mitochondria have a huge capacity to accumulate calcium and the permeability transition pore (PTP) complex is activated by calcium. When calcium levels markedly increase, the PTP complex can enter an irreversible high conduction state which dissipates the mitochondria membrane potential ultimately leading to cytochrome c release and initiation of apoptosis. We were interested to see if caspase activation was calcium-dependent. Chelators of calcium (EGTA and BAPTA) were used to prevent calcium effects and caspase activation was analyzed using the cell permeable, irreversible pan-caspase inhibitor, z-VAD-fmk. Ionomycin increased Ca²⁺ 3.1-

fold and ten 300ns pulses at 60 kV/cm increased calcium nearly 2-fold using fluo-3 as a calcium indicator. Ionomycin, a calcium ionophore did not activate platelets above control levels. These same pulses increased the presence of active caspase by about two fold and increased the number of caspase positive cells from 20% in control cells to about 80% in nsPEF-treated cells. The presence of EGTA and BATA had no effect on the presence of active caspases.

In a more recent study with E4 squamous carcinoma cells roles for calcium were more specifically identified [Ren and Beebe, 2011]. When conditions were sufficient to kill about 95% of the E4 cell population (ten 300ns pulses at 60kV/cm), cytochrome *c* release and cleavage of the BH3 only protein Bid to t-Bid was only partially caspase-dependent. An analysis of effects of calcium on Bid cleavage using EGTA and BAPTA-AM to chelate extra- and intra-cellular calcium, respectively, determined that intracellular calcium as an intrinsic mechanism was responsible for about 30% of calcium-dependent Bid cleavage and extracellular calcium as an extrinsic mechanism was responsible for about 70% of calcium-dependent Bid cleavage. This study also observed that nsPEFs activated calpains in a calcium-dependent manner, but experiments did not rule out involvement of other proteases. The results indicated that multiple mechanisms were involved in Bid cleavage and cytochrome *c* was released and calcium was mobilized from intracellular and extracellular sources. A possible common mechanism could be formation of nanopores in these membranes.

There are other possible effects of nsPEFs on the endoplasmic reticulum. It is highly likely that other endoplasmic reticulum responses to pulse power will occur especially under conditions that are below the threshold or just above the threshold for cell death when a population of cells will not survive. This is especially relevant given roles for the endoplasmic reticulum in protein translation and folding. Cells respond to stress through changes in gene expression and the regulation of protein levels can be modulated by the endoplasmic reticulum. Regulation of eukaryotic initiation factor-2alpha by phosphorylation and internal ribosome initiation through the internal ribosome-entry site are two examples for direct roles of the endoplasmic reticulum in translation control in cellular stress responses and apoptosis [Holcik and Sonenberg, 2005]. Stress responses from the endoplasmic reticulum can also occur by crosstalk with the mitochondria to induce cytochrome *c* release or through caspase-12, which is transported to and/or located in the mitochondria and can act without cytochrome *c* release [Momoji, 2004; Szegezdi et al., 2003]. Under these conditions, the downstream responses of endoplasmic reticulum stress include a mitochondria-mediated response, often involving calcium mobilization or a mitochondria-independent response that involves the activation of caspase-12 and caspase-4. Both of these "pathways" lead to the activation of executioner caspases. Effects of nsPEFs in the stress response, control of translation and caspase activation through effects on the endoplasmic reticulum have not been investigated.

7. Effect of nsPEFs on mitochondria

Mitochondria are often referred to as the cell's "power plant" since they are responsible for production of ATP. However, another important function of mitochondria is in programmed cell death. A primary focus here will be on the role of mitochondria in pulse power-induced cell death, which will be discussed with specific references to effects on ATP levels, mitochondria membrane potential and release of pro-apoptotic factors. Analyses of

nsPEF effects on mitochondria have only just begun by analysis in B16f10 melanoma [Ford et al., 2010] as well as E4 squamous cell carcinoma [Ren and Beebe, 2011]. Ford and co-workers [2010] recently demonstrated that nsPEFs induced a decrease in cellular ATP levels and decreased the mitochondria membrane potential in B16f10 melanoma cells. The loss of the mitochondria membrane potential is a common event during apoptosis. Figure 2 shows effects of nsPEFs on the mitochondria membrane potential in B16f10 melanoma cells.

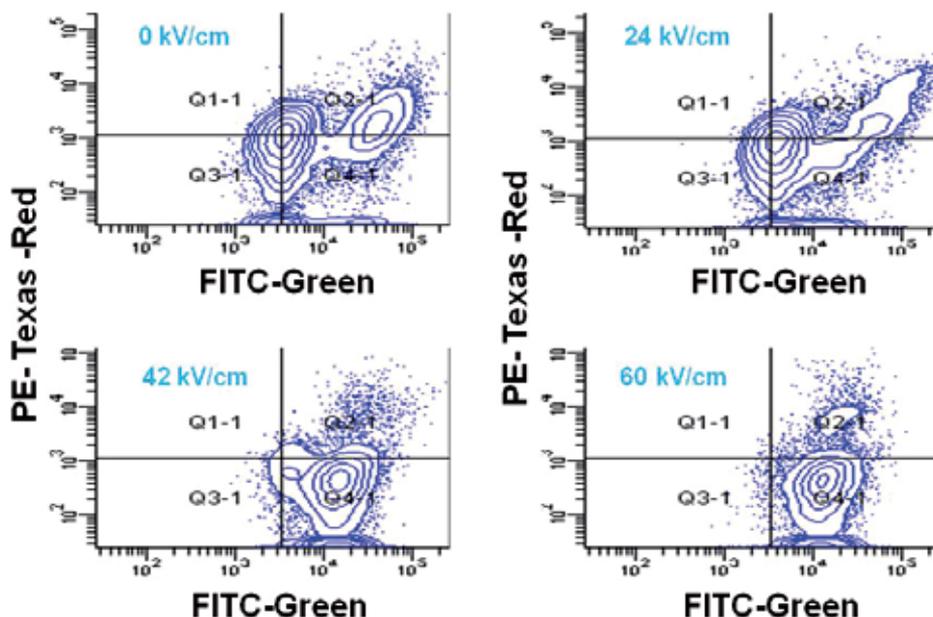


Fig. 2. nsPEFs decreases the mitochondria membrane potential in B16f10 cells.

In non-apoptotic cells, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) exists as a monomer in the cytosol as a FITC fluorophore as shown through the green channel on the X-axis. It also accumulates as aggregates in the mitochondria which stain red as shown through the PE-Texas Red channel on the Y-axis. In apoptotic and necrotic cells, aggregated, red JC-1 decreases and monomeric, green JC-1 increases. The top left panel of Figure 2 shows control cells in two populations with different green monomeric JC-1 intensities. As nsPEFs are applied to the cells with increasing electric fields, red JC-1 aggregate intensities decrease (Y-axis) and green JC-1 monomer intensities increase (X-axis). The decrease in the mitochondria membrane potential occurred as quickly as it could be measure by flow cytometry (minutes). This transformation occurs in an electric field-dependent manner indicating that electric fields caused a decrease in the mitochondria membrane potential. As expected, the levels of ATP drop significantly in a similar manner [Ford et al., 2010]. Given that nsPEFs cause nanopore formation in the plasma membrane and also have effects on intracellular membranes it is possible that the electric fields cause nanopore formation in the inner mitochondria membrane resulting in a decrease in the potential across the membrane. Another possibility is that nanopore formation in the plasma membrane causes an increase in sodium in the cytosol, which causes the decrease in the mitochondria membrane potential. Further experimentation will be required to differentiate between these two possibilities.

Another common event during apoptosis is the release of pro-apoptotic factors from the mitochondria. Several methods are available to analyze cytochrome *c* release including immunoblots using mitochondrial-free cytosolic fractions [Beebe et al., 2003a]. Using this assay it is more difficult to quantify cytochrome *c* release and it is not possible to determine the number of cells in a population that release cytochrome *c*. Another approach is to use fluorescent antibody detection of cytochrome *c* by fluorescent microscopy and/or flow cytometry.

Cytochrome *c* release using immunoblot analysis was demonstrated in Jurkat cells exposed to nsPEFs [Beebe et al., 2003a]. Cytochrome *c* release occurred within 30-45 minutes after treatment and was coincident with caspase activation, which was determined with the cell permeable irreversible inhibitor z-VAD-fmk. nsPEFs also initiated cytochrome *c* release in HCT116 colon carcinoma cells, albeit it did not occur until the second hour, which was after active caspases were present. In B16f10 melanoma cells cytochrome *c* release was analyzed as well as release of the pro-apoptotic factors Smac/Diablo and apoptosis initiating factor (AIF). These results are shown in Figure 3 (below).

Unlike all other cells tested for cytochrome *c* release in response to nsPEFs, and in contrast to ethanol treated cells, B16f10 cells did not release cytochrome *c* while active caspases were detected using z-VAD-fmk [Ford et al., 2010]. Cells in experiments shown in Figure 3 were analyzed 3 hours after treatment. However, when analyzed between 1 and 7 hours after treatment, cytochrome *c* released was not detected. In data not shown, a second assay was used that was based on a loss of cytosolic cytochrome *c* from permeabilized cells before analysis with fluorescent antibodies (Innocyte assay, Cal Biochem) based on the procedure described by Waterhouse and Trapani [2003]. We were unable to detect cytochrome *c* release using this assay while about 50% of E4 squamous carcinoma cells released cytochrome *c* 1 hour after treatment with nsPEFs [Ren and Beebe, 2011]. However, as indicated in Figure 3, we did detect small increases in fluorescence with antibodies to Smac/Diablo and apoptosis initiating factor (AIF), albeit in small populations of cells (10-15%). Given that cytochrome *c* release was detected in Jurkat cells coincident with activation of caspases [Beebe et al., 2003a] as well as in HCT 116 cells [Hall et al., 2007a] and E4 squamous carcinoma cells [Ren and Beebe, 2011] after caspase activation, this suggests that nsPEFs have cell type-specific effects on mitochondria-mediated events associated with apoptosis-like mechanisms.

8. Effects of nsPEFs on nucleus / DNA

Possible effects of nsPEFs on the nucleus have been of specific interest since the technology was applied to cells and tissues. Given that these effects on cells and tissues are high in power and low in energy density, it would be expected that the energy deposited into cells from these electric fields would not be sufficient to break hydrogen bonds, especially those in DNA. However, some experimental data suggests that this might not be true in cells *in vitro* and tumor tissues *in vivo*. Fibrosarcoma tumors grown in mice and treated with nsPEFs *ex vivo* exhibited DNA damage using TUNEL analysis [Beebe et al., 2002, 2003b]. Stacey and colleagues [2003] evaluated possible genotoxic stress effects of ultra-high pulse power using nanosecond pulsed electric fields on 11 suspension and adherent cell lines. They evaluated cell survival assessed by clonogenic formation or live cell counts; DNA damage was determined by the comet assay and chromosome aberrations and cell cycle parameters by measuring the mitotic indices of exposed cells. Not all cell types were affected in the same

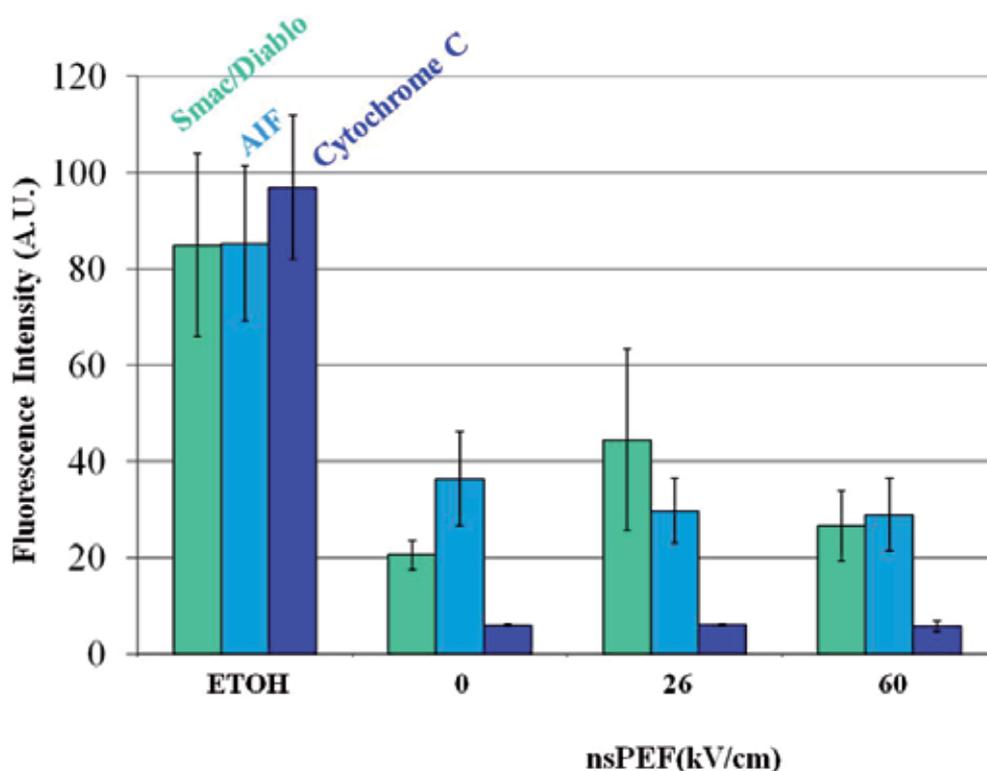


Fig. 3. NsPEFs have minimal effects on release of pro-apoptotic factors from mitochondria in B16f10 melanoma cells.

ways. After one 60 ns pulse with an electric field intensity of 60 kV/cm, non-adherent cultures exhibited a rapid decline in cell viability (90%), DNA damage, and a reduction in the number of cells reaching mitosis. Adherent cultures did not exhibit these effects under the same conditions with the exception of mouse 3T3 cells, which behaved as the suspended cells did. These results suggested that pulse-power-induced genotoxicity may be cell type-specific and therefore have possible applications in the selective removal of one cell type within a heterogeneous population of cells such as in diseased states. The comet assay also suggested possibilities for DNA damage in B16 cells *in vitro* when treated with nanosecond pulsed electric fields [Nuccitelli et al., 2009].

Another approach to identify effects on DNA was to use phosphorylation of Histone 2AX to identify possible DNA double strand breaks using fluorescent antibodies by flow cytometry or fluorescent microscopy. When DNA double strand breaks occur, Histone 2AX, a histone variant, is phosphorylated on Serine 139 and serves as a sensitive and early monitor to identify these events [Bonner et al., 2008; Rogakou et al., 1999]. When B16f10 cells were exposed to 300ns pulses at 60kV/cm, it was not possible to determine an increased Histone 2AX phosphorylation. While there were significant levels of phosphorylated Histone2AX, there were no significant differences between control and treated cells, even though >95% of treated cells were eliminated 24 hours later [Ford et al., 2010]. This suggests that DNA double strands breaks are not uncommon in control B16f10 cells. However, results were different in HCT116 cells.

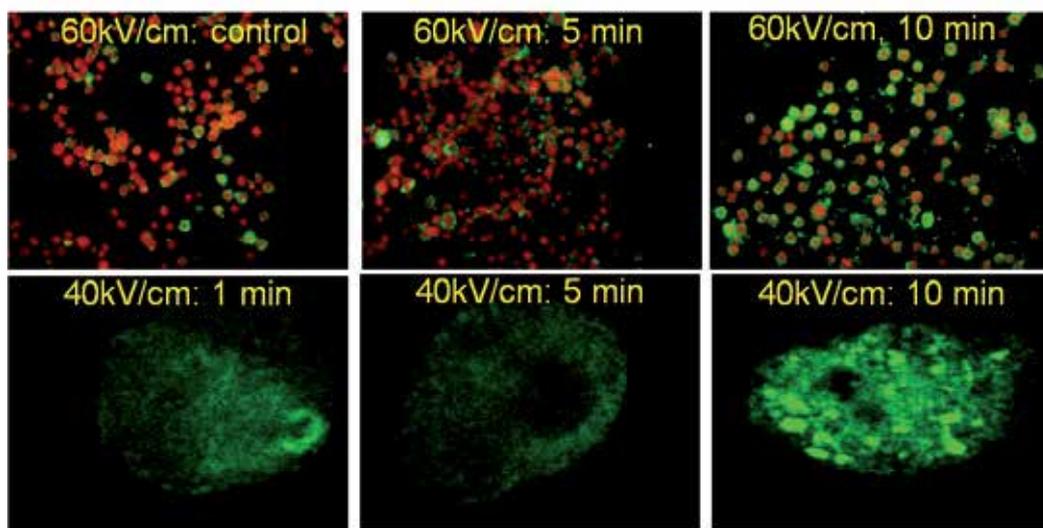


Fig. 4. nsPEFs induce DNA double strand breaks in HCT 116 colon carcinoma cells.

HCT116 colon carcinoma cells were exposed to ten 300ns pulses at various electric fields and analyzed for Histone 2AX phosphorylation with fluorescent antibodies specific for phosphorylation at the serine 139 site. Figure 4 (above) shows identification of phosphorylated Histone 2AX in HCT116 cells treated with ten 300ns pulses at 40 or 60 kV/cm. The top panels show 20x magnifications of cells treated with 60kV/cm, a condition that resulted in death of >95% of cells. Within 5-10 minutes HCT116 cells were positive for phosphorylation of Histone 2AX. The bottom panels shows 60x oil magnifications of representative HCT116 cell nuclei that were phosphorylated at Serine 139. The rapidity of phosphorylation was surprising if nsPEFs did not have direct effects on DNA. A common mechanism for DNA damage in response to ionizing radiation is the generation of reactive oxygen species. However, we were unable to detect reactive oxygen species in B16f10 cells treated in the same way [Ford et al. 2010]. These results were consistent with results of DNA damage using the comet assay, which identified rapid DNA damage [Stacey et al., 2004; Nuccitelli et al, 2009]. In experiments analyzing nsPEF effects on DNA double strand breaks in Jurkat cells that resulted in >95% cell death, Histone 2AX phosphorylation occurred several hours after treatment and was caspase-dependent, suggesting that this DNA damage was due to apoptosis [Ren and Beebe, unpublished results]. Taking all of this data together, it appears that nsPEF-induced DNA damage is cell type specific.

More general nsPEF effects on cell nuclei in HL-60 cells were determined in cells when non-lethal pulse power conditions caused the nucleus in acridine orange-stained HL-60 cells to become irregularly shaped as the fluorescence decreased [Chen et al., 2004]. When HCT116 cells were treated with non-lethal pulse power using nanosecond pulsed electric fields, reversible changes in nuclear size and morphology were observed indicating effects on nuclei even under conditions that resulted in cell survival [Hall et al., 2005]. In a different approach to analyze nsPEF effects on cell nuclei, Chen and co-workers [2007] used confocal microscopy and flow cytometry to observe Smith antigen antibody (Y12) binding to nuclear speckles, known as small nuclear ribonucleoprotein particles (snRNPs) or intrachromatin granule clusters (IGCs), in Jurkat cells following one or five non-lethal 10ns pulses at 150

kV/cm. These experiments indicated that nsPEFs disrupted pre-messenger RNA splicing mechanisms but did not allow propidium uptake, suggesting the nuclear effects occurred in the absence of plasma membrane pores larger than about a nanometer. Furthermore, these effects were cell cycle dependent. When cells were synchronized to the G2-M phase with nocodazole, exposing cells in the mitotic phase to five consecutive 10ns pulses immediately and significantly increased the number of nuclear speckled substructures, suggesting direct effects to inhibit RNA transcription mechanisms. While these pulse power conditions resulted in significant cell survival, the long term effects after these responses have not been analyzed.

NsPEF effects reviewed thus far have been with B16f10 cells and other cell types *in vitro*. Effects on DNA in B16f10 tumors in mice have been analyzed [Chen et al., 2010]. In these studies, hairless female SKH-1 mice were injected subcutaneously with 1×10^6 B16-F10 cells. Tumors developed within 8-10 days. B16f10 tumors were treated *in vivo* with one hundred 300ns pulses at 40kV/cm and analyzed for phosphorylated Histone 2AX, 1, 3, 6 and 24 hours after treatment. The results indicated a time-dependent increase in γ H2AX in melanoma, with significant differences occurring at 1 hour (15% of cells), reaching a peak at 3 hours (85% of cells) and decreasing to control levels thereafter. These transient peaks in Histone 2AX phosphorylation coincided with TUNEL positive cells and pyknotic nuclei. Quantitative differences were observed by calculating mean nuclear area (μm^2) between control and treated tumor nuclei during the first 24 hours after treatment with nsPEFs. Treated tumor nuclei were significantly smaller than control nuclei. The transient nature of histone 2AX phosphorylation and TUNEL positive cells suggested that DNA repair was initiated but not completed. When DNA was analyzed by agarose gel electrophoresis large DNA fragments, but not 180bp fragmentation ladders, were observed. The presence of active caspases peaked after peaks of histone 2AX phosphorylation and TUNEL positive cells. Taken together, these results suggested that the DNA damage occurred by a caspase-independent mechanisms and that apoptosis did not go to completion (see nsPEF effects on apoptosis mechanisms, below).

Most data for effects of pulse power-induced effects on DNA in cells and tissues suggests that at least some DNA damage may be caused by direct nsPEF effects. DNA damage in B16f10 melanoma cells and tumors does not appear to be a caspase-associated apoptosis marker. However, the mechanism(s) remain to be defined. Pulse power is not expected to generate sufficient energy to break hydrogen bonds and it may not be expected to generate reactive oxygen species through ionization of water. However, reactive oxygen species can be generated by effects on mitochondria, which are clearly present after pulse power treatment of B16f10 and other cells. Nevertheless, pulse-power induced reactive oxygen species in B16f10 cells [Ford et al., 2010] or E4 squamous carcinoma cells [Ren and Beebe, 2011] *in vitro* were not detected when CM-H₂DCFDA was used as a reactive oxygen species marker. Since ATM (ataxia telangiectasia mutated) kinase and/or ATR (ATM and Rad 3-related) kinase are activated with ionizing radiation and UV light, it would be of interest to carry out kinetic analysis of these kinases after cells are exposed to pulse power and correlate this with histone 2AX phosphorylation.

9. Effect of nsPEFs on actin cytoskeleton

The cytoskeleton forms a dynamic network of filamentous protein structures that crisscrosses the cytoplasm, providing shape, mechanical support, modes for intracellular

transport of synthesized proteins and the capacity for motility. It is contiguous with the plasma membrane. Given that nsPEFs have effects on plasma membranes and intracellular structures and the cytoskeleton is an extension of the plasma membrane, it was likely that nanosecond pulsed electric fields would affect the cytoskeleton.

Effects of pulse power ablation on the actin cytoskeleton have been demonstrated [Hall et al., 2007a]. Human HCT 116 colon carcinoma cells were synchronized to S phase (95%) by thymidine block or analyzed unsynchronized (50% S phase). The actin cytoskeleton was labeled with rhodamine phalloidin and pulsed with three 60ns pulses at 60kV/cm and visualized by fluorescent microscopy. Both S-phase synchronized and unsynchronized control cells exhibited well-defined peripheral cytoskeletal structures around large nuclei. One hour after treatment, the cytoskeletal structure of unsynchronized cells exhibited a more random, less organized structure near the plasma membrane with blebbed-like structures. In contrast, the cytoskeletal structure of S phase cells exposed to the same pulses was not significantly perturbed. Five hours after treatment with these conditions, control and treated cell were indistinguishable with 90-95% survival [Hall et al., 2007a]. When these cells were pulsed with three 300ns pulses at 60kV/cm, which killed 90% of cells, S-phase cells and unsynchronized cells exhibited rearranged actin cytoskeletons.

These experiments demonstrated several new facts about nsPEF effects on actin and how actin functions in HCT 116 cells. First, nsPEFs have differential cytoskeletal effects on cells in S phase, but no differential effects on survival. A second aspect is the capacity of the actin cytoskeleton to resist effects of nsPEFs during DNA synthesis. This is contrary to the idea that proliferating cells, with potentially vulnerable DNA in the absence a nuclear membrane, would be more susceptible to nsPEFs. Third, when nsPEFs perturb the actin structure with low level pulse power (three 60kV/cm pulses at 60 or 300ns), there is a transient influence that allows recovery between 1 and 5 hours after treatment with cell survival. However, when pulse power conditions are sufficiently intense, cells cannot survive the exposure. In these studies, two nsPEF thresholds are demonstrated. One threshold is for pulse power intensity that supersedes the capacity of actin cytoskeleton to resist rearrangement during DNA synthesis in S-phase and a second threshold is pulse power intensity that surpasses the capability of cells to survive nsPEF ablation. This suggests that in some tumors this treatment could complement chemotherapeutic agents such as vincristine, vinblastine and other vinca alkaloids that bind to tubulin and prevent polymerization as well as paclitaxel and other taxanes that bind to tubulin and prevent depolymerization.

Effects of nsPEFs on B16f10 cell actin cytoskeletal structures using ten 300ns pulses at 60kV/cm, like that used in HCT 116 cells had unnoticeable effects on the cytoskeleton [JA Liu and SJ Beebe, unpublished]. This again suggests cell type-specific differences. This may not be too surprising since pulse power-induced plasma membrane effects were different between B16f10 and HCT116 cells as well as other cells. However, Ford et al. [2010] observed that when active caspases were expressed in B16f10 cells, the actin cytoskeleton was not readily observed 2-3 hours after pulse power treatment. Since actin is a caspase substrate, this suggested that the actin cytoskeleton is dismantled by active caspases after pulse power treatment.

10. Effect of pulse power on apoptosis-like cell death mechanisms

The landscape for defining cell death mechanisms has become more complex as our understanding of life and death advances. Since apoptosis initiation and progression are complex processes and are cell type-specific, apoptosis mechanisms and pathways are often

simplified by classifying pathways as intrinsic and extrinsic. The extrinsic pathways is further classified as type I cells that do not use mitochondria cytochrome *c* release and type II cells that do [Fulda and Debatin, 2006; Lavrik, 2010]. The fate of cells depends on a set of sensors and positive and negative regulators whose balance will determine cell fate and whether apoptosis is initiated or not. The mitochondria play major roles in these mechanisms. There may be no linear apoptosis pathways, but grouping apoptosis into intrinsic mechanisms, which are linked to the mitochondria, and extrinsic mechanisms, which are linked to death receptor complexes in the plasma membrane, provides some structure for discussion and analysis. There also appears to be an intrinsic mechanism that originates in the endoplasmic reticulum and may or may not be linked to the mitochondria. The intrinsic pathway is regulated through pro- and anti-apoptotic Bcl-2 family members in response to intracellular stresses with mitochondria-dependent release of cytochrome *c* and other factors causing activation of apical initiator caspase-9 through formation of the apoptosome with APAF-1. This then leads to activation of downstream execution caspases - 3, -6, and/or -7. The regulation of mitochondria-dependent mechanisms is complex. Activators of this pathway include endoplasmic reticulum stress, as discussed earlier, DNA damage, hypoxia, and reactive oxygen species and growth factor deprivation, among others. Caspase activation is controlled by inhibitors of apoptosis (IAP), which can be inactivated by pro-apoptotic factors released from the mitochondria such as Smac/Diablo and Omi/OtrA. P53, the "guardian of the genome" senses potential apoptotic signals and leads to increases in factors such as Puma, Nova, and Bax, which lead to release of cytochrome *c*. However, these factors are opposed by anti-apoptotic factors such as Bcl-2 and Bcl-xl.

The extrinsic pathway is initiated by death receptor ligands that bind and trimerize death receptors such as Fas, TNF or TRAIL at the plasma membrane. This signals the recruitment of intracellular adaptor proteins and the apical initiator caspase-8 to the plasma membrane and the formation of a death-induced signaling complex (DISC). In type I cells, caspase-8 directly activates the executioner caspase-3. The formation of DISC is also regulated by positive (caspase-8) and negative (FLIP) regulators. In some cells (type II), formation of the DISC is insufficient to signal caspase-8 and caspase-3 directly. Instead caspase-8 cleaves a BH-3 only Bcl-2 pro-apoptotic protein Bid, forming a truncated Bid (t-Bid) that signals through the mitochondria, releasing cytochrome *c* causing activation of caspase-9, like that occurring in the intrinsic mechanism. In this way, the extrinsic pathway is connected to the intrinsic pathway through mitochondria-mediated mechanisms in type II cells.

Mechanisms for nsPEF-induced apoptosis induction appear to be dependent on the pulse conditions and/or on cell type. For example, pulse power-induced Jurkat cell apoptosis, in response to three 60ns 60kV/cm pulses, involved coincident release of cytochrome *c* and activation of caspases within the first 30-45 minutes after pulse delivery [Beebe et al., 2003a]. However, it is not yet clear if this mitochondria-dependent response occurs through the intrinsic pathway due to intracellular effects on the endoplasmic reticulum or mitochondria or occurs through the type II cell extrinsic pathway. In contrast, nsPEF-induced apoptosis in HCT116 colon carcinoma cells involves caspase activation in the first 45-60 minutes post-pulse with cytochrome *c* release as a later event [Hall et al., 2007a].

When B16f10 cells were suspended in Dulbecco's PBS (DPBS) solution and exposed to ten 300ns pulses with increasing electric fields, an increasing number of cells exhibit active caspases as determined 1 hour after treatment using the cell permeable pan caspase irreversible inhibitor z-VAD-fmk [Ford et al., 2010]. This is opposed to elevating caspase

levels in a given population of cells. Thus this increase in the numbers of cells with active caspases occurred in an electric field-dependent increments. About 10% of cells were positive for active caspases in untreated cells and that number increased to greater than 75% when treated with ten 300ns pulses at 60 kV/cm. The analysis by flow cytometry indicated an increasing population of cells shifting to the right with increased fluorescence with the FITC- labeled pan caspase probe. This indicates that cells become positive for this probe in an all or none manner. This is consistent with the concept that once caspases are active they reach a point-of-no-return with a positively reinforced cascade of caspase activation leading to cell death. In addition, this behavior indicates that the B16f10 cell population responded in a heterogeneous manner with only a subpopulation of cells showing positive responses with each increasing electric field increment. Also when cells were treated with 20% ethanol they become positive for active caspases.

In another experimental approach, cells were treated as in Figure 5 with ten 300ns pulses at 60 kV/cm and analyzed by fluorescent microscopy. DAPI was used to identify the nuclei and a cell permeable, irreversible inhibitor or pseudosubstrate (Sulforhodamine-DEVD-fmk with red fluorescence) was used to identify caspase-3/7 (Figure 5). In controls that were sham treated without exposure to electric fields (0 kV/cm), cell nuclei were stained blue with DAPI, but caspase-3/7 cells were essentially absent {or in few cells (<10%)} and therefore exhibited little of no red fluorescence. Cells that were treated with nsPEFs ultra-high pulse power as indicated above exhibited a large population of red fluorescence with the presence of active caspase-3/7.

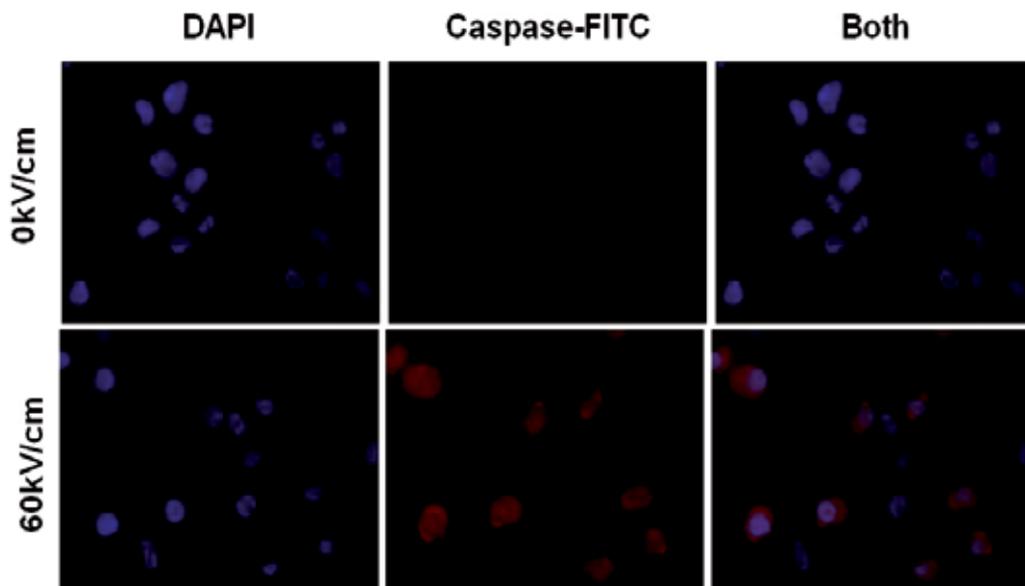


Fig. 5. nsPEF treated b16f10 cells exhibit increased binding of a cell permeable, irreversible caspase inhibitor.

Based on available evidence, it appears that nsPEF-induced apoptosis in B16f10 cells mimics the extrinsic pathway because active caspase-3-7 were present without concomitant cytochrome *c* release from B16f10 cells. Thus, pulse power induced apoptosis in these cells

by mechanism(s) that were mitochondria-independent. Of interest here is the possibility for these pulses to activate mechanisms that bypass mutations that are common in cancer and resistances to treatment through mutations in mitochondria-mediated apoptosis mechanisms. For example [see Soengas and Lowe, 2003 for a review], Bcl-2 is often upregulated in melanoma to protect cells from apoptosis by inhibiting cytochrome *c* release from mitochondria. In addition, MitF is a factor that may also contribute to melanocyte survival by the transactivation of Bcl-2, which supports melanocyte survival. In addition, Apaf-1 protein and mRNA expression are frequently downregulated in metastatic cell lines and tumor specimens. Neither of these mutations would deter nsPEFs from preventing melanoma ablation. Thus, there may be advantages for applying pulse power ablation for eliminating melanoma at early stages (see below).

11. Multiple mechanisms for nsPEFs to eliminate cancer cells *in vitro*

Effects of nsPEF conditions have been observed that are coincident with a number of cellular responses from plasma membranes, intracellular cell membrane, proteases, and Bcl-2 family member proteins. Cell responses that seem to happen rapidly are likely direct actions of membrane charging and/or energy density “dose” effects are decreases in the plasma cell membrane potential as well as the mitochondria membrane potential. Phosphatidylserine externalization can also occur as a direct effect of pulse power. The best explanations for these phenomena are the formation of nanopores in those membranes. Likewise, increases in intracellular calcium occur by mobilization from extracellular and intracellular sources, which are relatively rapid and sensitive responses that could also be due to nanopores in the plasma membrane and endoplasmic reticulum. Other pulse-power-induced cellular responses include cytochrome *c* release, cytoskeletal changes, disruption of pre-messenger RNA splicing mechanisms, changes in nuclear shape and morphology, DNA double stranded breaks and general DNA damage, depending on pulse power conditions and the cell type. Other changes include increases in active caspases and calpains, cleavage of Bid to t-Bid and changes in other Bcl-2 proteins. There appears to be different thresholds and some of these changes appear to be dependent on the cell type. Under intense pulse power conditions, cells die by different mechanisms that may or may not be related to programmed cell death such as apoptosis and other death responses may be due to caspase-independent mechanisms including necrosis. Since many of these responses have been observed in different cell types, under different pulse power conditions and cell concentrations, in different buffers and by different assay procedures, it is difficult to determine specific cell death mechanisms. Just as difficult to determine is whether cell death is due to direct electric field effects or subsequent biological effects and whether cell death or sub-lethal effects are due to plasma membrane or intracellular membrane charging or energy density-related effects. Based on our present understanding of these numerous complex events, it is safe to conclude that given the plethora of cell responses that cell death is due to multiple mechanisms that depend on a variety of conditions. Given these complex scenarios, it could be simply proposed that when pulse durations are short and electric fields are low a minimal number of cellular “targets” respond. As the pulse durations become longer and electric fields higher, increasing numbers of “targets” respond. Where these targets are located and how sensitive they are or what their thresholds are appears to be dependent on the cell type.

12. Multiple mechanisms for nsPEFs to eliminate tumors *in vivo*

Treatment of melanoma requires new modalities to those presently available. Presently available melanoma therapy has significant limitations due to poor efficacy, quasi-tolerable toxicity and limited enhancement in survival and life quality. Here we discuss encouraging successes in applications of nsPEF for the treatment of cancer. *In vitro*, pulse power ablation induces cell death through multiple mechanisms that appear to be cell type-dependent. As discussed above, effects are evident on the plasma membrane, endoplasmic reticulum, cytoskeletal structure, mitochondria and nucleus. Coincident with cell death are events that are dependent and independent of calcium, caspases and cytochrome *c* release depending on the cell type. In murine B16f10 melanoma *in vitro*, nsPEFs induce caspase activation without cytochrome *c* release and with limited effects on phosphatidylserine externalization. The first studies demonstrating the possibility that nsPEFs could kill cancer cells used a B10.2 fibrosarcoma tumor in mice [Beebe et al., 2002, 2003b, 2004]. These studies used the first developed electrode design for *in vivo* applications of pulse power to tumors. Since then significant advances with a number of electrode designs have been used [Beebe et al., 2010; Kolb et al., 2009; Garon et al., 2007; Nuccitelli et al., 2006, 2010]. The fibrosarcoma studies followed studies showing that pulse power with nanosecond pulsed electric fields induced markers for apoptosis in Jurkat and HL-60 cells [Beebe et al., 2002]. In initial fibrosarcoma tumor studies, nsPEFs reduced tumor size *in vivo*, induced activation of caspase catalytic activity and demonstrated the presence of TUNEL positive cells when tumors were treated *ex vivo* [Beebe et al., 2003b, 2004]. Since then, nsPEFs were shown to eliminate B16f10 melanoma tumors *in vivo* without recurrence using two different electrode designs [Nuccitelli et al., 2006, 2009]. These studies demonstrated calcium mobilization and confirmed the possibility for DNA damage using the comet assay in B16f10 cells *in vitro* and decreased microvascular density using CD34 as a marker *in vivo*. Chen et al. [2010] revealed apoptosis initiation using active caspase-specific antibodies and the expression of TUNEL positive cells confirming DNA damage *in vivo*. In addition, this study also showed that nsPEFs could induce DNA double strand breaks using antibodies specific for phosphorylated Histone 2AX, an early and sensitive marker for this trait [Bonner et al., 2008]. These DNA damage and DNA double strand break markers peaked before caspases were fully active, suggesting that they might not be due to apoptosis. This study also indicated that nsPEFs may be responsible for anti-angiogenesis mechanisms showing decreases in vascular endothelial cell growth factor (VEGF), which is required for the angiogenic switch, a limiting factor for multistage carcinogenesis [Hanahan and Weinberg, 2000, 2011] and platelet derived endothelial growth factor (PD-ECGF). Decreases in several microvascular density factors, including CD-31, CD-34 and CD-105, were also demonstrated. CD-31 (PECAM-1), a platelet-endothelial cell adhesion molecule used as a pan-endothelial cell marker, and CD-34, an endothelial cell marker, were decreased by 65-70%. CD-105 (endoglin) was decreased >40%. CD-105, which is part of the TGF β receptor complex, is an important angiogenic factor that is strongly expressed in tumors and is an independent prognostic indicator, wherein increased MVD correlates with shorter survival [Duff et al., 2003].

The results indicate that pulse power ablation target two of the seven common cancer hallmarks (Hanahan and Weinberg, 2000, 2011; Kroemer and Pouyssegur 2008) on solid B16f10 melanoma tumors, including apoptosis evasion and sustained angiogenesis. The latter is critical for a third hallmark, invasion and metastasis. The study concluded that

apoptosis was initiated but most likely did not go to completion as suggested by the absence of DNA fragmentation ladders but the presence of large molecular weight DNA fragments on agarose gels [Chen et al., 2010]. It was suggested that the initiation of apoptosis without completion was likely due to loss of vascular viability contributing to infarctive tumor death. However, the presence of active caspases for several hours after nsPEF treatment could help disassemble tumors to initiate the removal of dead tumor cells. Consequently, nsPEF ablation induces B16f10 tumor elimination by multiple mechanisms that can bypass common cancer mutations that frequently result in chemotherapeutic resistances and metastasis. The application of nsPEF ablation is safe, has no systemic side effects, is non- or minimally invasive, leaves no scars, and provides an inexpensive and effective method to the arsenal for cancer treatment strategies [Chen et al., 2010; Nuccitelli et al., 2006, 2009, 2010]

13. Advantages for nsPEF ablation as a cancer therapy

There are a number of advantages for using nsPEF ablation as a means for cancer therapy as opposed to other physical methods that rely on overt necrosis for tumor cell death. These advantages include (1) targeting multiple programmed cell death mechanisms including apoptosis induction and anti-angiogenesis, two well known cancer hallmarks, the latter necessary for a third cancer hallmark, invasion and metastasis; (2) targeting rapid death induction with minimal treatment exposures, which reduces chances for resistances and recurrences; (3) targeting non-mitochondria-mediated programmed cell death in melanoma, which can bypass many melanoma and other cancer-causing mutations; (4) an apparent broad specificity for cell death induction, effective for all cells within electric fields, including rapidly growing tumor cells, slower growing host cells that have been hijacked by tumors and cancer stem cells, all constituting the tumor mass and the microenvironment; (5) small vessel, local infarction, which deprives tumors of feeder vessels that are important for immediate oxygenation and nutrition, which provides local stresses; (6) minimal local and systemic side effects and (7) the potential for enhancing immune surveillance from cells undergoing apoptotic.

NsPEF ablation provides a local targeted treatment at the level of the entire tumor without systemic effects, affecting multiple molecular structures and functions in plasma membranes and intracellular organelles. All tumor cells exposed to conditions of pulse duration, number and electric field that are above the threshold for cell death are subject to programmed and other forms of cell death. The foremost targets bypass two important hallmarks of cancer causing apoptosis-like appearances and anti-angiogenesis. In its full capacity, this should lead to inhibition of invasion and metastasis, another cancer hallmark. The multi-mechanisms for nsPEF interactions with tumors are similar to using a combination of at least two chemotherapeutic agents and/or molecular targeted drugs that induce apoptosis-like characteristics and limit angiogenesis; both well defined sites for cancer targeted drugs. The observed decreases in vessel numbers and angiogenic factors (VEGF and PD-ECGF) prevent the possibility for re-vascularization and reduce chances for tumor cells to continue to proliferate [Chen et al., 2010]. Sustained hypoxia has been implicated in metastasis and hypoxia induced factor (HIF) transcriptional activity that is beyond that of normal tissue [Cairns et al., 2001; Peng et al., 2006]. The combination of apoptosis-like qualities and anti-angiogenesis as sites of nsPEF action makes this an attractive cancer therapeutic modality [Chen et al., 2010].

Another advantage to nsPEF interactions with tumors is the rapid onset of apoptosis-like features and some level of tumor infarction. Caspase activation *in vitro* is seen within 30-45 minutes [Beebe et al., 2002, 2003a] and within the first hours after treatment *in vivo* [Chen et al., 2010]. This rapid caspase activation is likely to rapidly induce cell death mechanisms. In contrast, chemotherapeutic agents, ionizing radiation and molecular targeting drugs are administered over weeks or months and often do not eliminate cancer but reduce tumor size or stabilize it. This provides a potential for mechanisms to allow tumor cells to escape therapeutic action and increases the possibility for treatment resistances and recurrences. Examples include upregulation of drug efflux transporters and tumor immune evasion in chemoresistant melanomas [Schatton et al., 2009] and the chemotherapy-induced upregulation of factors like clusterin, an anti-apoptotic protein conferring resistances to several cell death agonists [Wei et al., 2009]. As indicated earlier, two mechanisms of resistance to PLX4220 treated melanoma tumors include creating alternative survival pathways by overexpressing a cell surface Beta-type protein platelet derived growth factor receptor or by reactivating the normal BRAF survival pathway [Nazarian et al., 2010]. Ultra-high pulse power-induced interruption of the tumor small vessel blood supply is also rapid, limiting blood flow to the tumor as it is being dismantled, at least in part by apoptosis-like mechanisms. nsPEF ablation has rapid therapeutic onset, which should reduce the potential for resistances and recurrences as all tumor cells are affected by conditions above the threshold for cell death.

Many mutations that lead to cancers often occur in mitochondria-mediated mechanisms and pathways, most likely because there are more regulatory sites through intrinsic and Bid-dependent extrinsic pathways than in mitochondria-independent apoptosis pathways [Hanahan and Weinberg, 2011]. Consequently, many chemotherapeutic agents and ionizing radiation have significant effects on mitochondria-dependent apoptotic mechanisms [Latai, 2008]. nsPEF ablation has both mitochondria-dependent and -independent sites of action that appear to be cell type-dependent. In melanoma, the apparent exclusive recruitment of cytochrome c-independent extrinsic mechanisms provides an alternative mechanism to many cancer therapeutic treatments that act on mitochondria-dependent pathways. A simple, bistable rate-equation-based model of apoptosis pathways predicted that the extrinsic caspase-8 mechanism was more sensitive than the mitochondrial intrinsic pathway for electric pulse induced cell apoptosis [Song et al., 2010], which is in keeping with results from B16F10 melanoma studies [Ford et al., 2010] as well as HCT116 colon carcinoma [Hall et al., 2007a] and E4 squamous cell carcinoma studies [Ren and Beebe, 2011]. Thus, by favoring the extrinsic apoptosis pathway, nsPEFs may bypass many cancer causing mutations in mitochondria-mediated apoptosis mechanisms, which are often involved in resistances and recurrences.

Another potential advantage of nsPEF ablation for cancer therapy is related to considerations for cell type specificity. Chemotherapeutic drugs and ionizing radiation primarily affect rapidly dividing cells. Effects of nsPEFs appear to be cell-type specific for a number of cell responses suggesting some cell type specificity; however, it remains to be determined if this has therapeutic relevance. Cultured cells that grow attached as opposed to cells in suspension require longer pulse durations, greater numbers and/or higher electric fields to elicit cell responses [Stacey et al., 2003], including cell death [Beebe et al., 2002; Hall et al., 2007a]. In contrast to conventional electroporation, which affects larger cells more readily than smaller ones, cell size did not matter for plasma membrane permeabilization

with nsPEF ablation [Hair et al., 2003]. However, there is no evidence that nsPEF ablation preferentially affect only rapidly proliferating cells. S phase synchronized cells under limiting pulse power conditions exhibited greater membrane integrity and maintained cytoskeletal structure but did not differ in survival compared to unsynchronized cells [Hall et al., 2007b]. Thus, within a heterogeneous tumor mass, nsPEF therapy is expected to induce cell death in rapidly proliferating tumor cells as well as slower proliferating host cells that are collaborating with tumor cells regardless of their size. This suggests an alternative to many therapeutic regimens that predominantly target rapid proliferating cells. Melanoma tumors also can contain cancer stem cells or other slower cycling cells, which possess characteristics common to normal stem cells, including self renewal capacity, high tumorigenicity and potential to differentiate into multiple cell types [Fang et al., 2005; Grichnik et al., 2006; Roesch et al., 2010; Zabierowski and Herlyn; 2008a, b]. Cancer stem cells or other slower cycling cells may be more prevalent in tumors than initially considered as demonstrated with melanomas from 12 different patients [Quintana et al., 2008]. Herlyn and colleagues have suggested an alternative to the unidirectional stem cell model in melanoma proposing a dynamic temporarily distinct subpopulation of slow cycling melanoma cells that are responsible for tumor maintenance [Roesch et al., 2010]. The existence of these slow cycling cells is clinically relevant because they would be less resistant to most therapeutic regimens; however, they would probably not be resistant to nsPEF ablation. Cancer stem cells or slow cycling cells have been reported to be responsible for recurrences after chemotherapy and ionizing radiation therapy through multiple mechanisms [Weissman and Clarke, 2009]. One of these mechanisms is to minimize therapy-induced DNA damage that is produced by free radical scavengers to minimize the effects of reactive oxygen species (ROS). Cancer stem cells had significantly lower levels of ROS and enhanced ROS defenses compared to non-tumorigenic cells [Diehn et al., 2009]. nsPEF ablation is non-ionizing and it does not appear to induce cell death by generating measurable ROS in B16F10 melanoma cells [Ford et al., 2010] or in E4 squamous cell carcinoma [Ren and Beebe, 2011]. Thus, this mechanism would not provide survival advantages to cancer stem cells exposed to nsPEF ablation. Another mechanism that may be responsible for resistance and recurrences with conventional treatments is to preferentially activate DNA damage checkpoint response and increases in DNA repair capacity [Bao et al., 2006]. nsPEF ablation does cause DNA damage in B16F10 melanoma cells [Nuccitelli et al., 2009] and tumors [Chen et al., 2010]. However, DNA damage may not be a major cause of cell death in these tumors. Furthermore, DNA damage induces apoptosis through release of pro-apoptotic factors from mitochondria [Gross et al., 1999; Hengartner et al., 2000; Korsmeyer et al., 2000] and nsPEF ablation induces melanoma cell death with minimal release of pro-apoptotic factors [Figure 3; Ford et al., 2010]. Thus, minimizing DNA damage and enhancing repair would not provide survival advantages to cancer stem cells or slow cycling cells exposed to nsPEF ablation.

An early study investigating local tissue effects at tumor treatment sites indicated that blood flow to the tumor was disrupted as blood cell leaked out of the tumor around small vessels [Nuccitelli et al., 2006]. This local tumor infarction with an absence of local blood flow for about two weeks appears to be sufficient to deprive tumors of needed oxygenation and nutrition to facilitate tumor demise. A subsequent analysis also demonstrated the presence of iron stain suggesting nsPEF caused slight hemorrhage in the treated tissue [Chen et al., 2010]. Other studies demonstrated that microvessel density markers were significantly

reduced, indicating that angiogenesis and/or vasculogenesis were significantly thwarted [Chen et al., 2010; Nuccitelli et al., 2009].

An important benefit to local treatment with nsPEFs is an absence of chemical side effects and toxicities, which are common with nearly all systemic treatments, especially chemotherapy and ionizing radiation. In studies with mice, nsPEF ablation has minimal and resolvable effects on skin. With parallel plate electrodes that eliminated B16F10 melanoma, the stratum corneum showed signs of necrosis and hemorrhage with accompanying superficial erosion of the epidermis [Nuccitelli et al., 2006]. However, these characteristics appeared two days after treatment, differentiating the effect from burn or heat related injuries, which occur immediately. With a four plus one needle array electrode, nsPEF ablation caused some edema and bleeding, but the damage was resolved within a week [Chen et al., 2009]. Small scabs formed but were resolved within two weeks and did not leave a scar. However, mice do not readily scar. In an unpublished clinical study observing effects of nsPEFs on human skin, treatments with two parallel needle electrodes caused some irritation, redness and itching at insertion / treatment sites, which were readily relieved by anti-histamines, local anti-inflammatory ointment and protection from scratching. The treatments caused no permanent scars or discoloration of skin regardless of pigmentation. While there was some pain and discomfort with applications of nsPEF without anesthesia, they were eliminated when a local anesthetic was injected at treatment sites. In addition, application of nsPEFs resulted in few/no muscle contractions, when applied appropriately, which are common with conventional electroporation and irreversible electroporation. In addition, studies monitoring general reactions to nsPEF ablation with parallel plate electrodes, mice had slightly higher heart rates and respiratory rates, but body temperature and systolic blood pressure did not change significantly [Chen et al., 2009]. Thus, as tested so far, applications of nsPEF ablation are generally safe, non-toxic and without scarring or other permanent effects on skin in mice and humans. While pulse power treatments can now be used for surface tumors using needle or plate electrodes, applications to internal tumors will likely be possible as catheter electrodes are developed for laparoscopic surgeries. For all nsPEF treatments, multi-needle electrode systems with adjustable field orientations would likely enhance apoptosis in the context of pulsed voltage-induced inactivation of tumor cells [Song et al., 2010].

An ending argument for applications of nsPEF ablation involves a significant question for the treatment of melanoma and other cancers: Can pulse power ablation with nsPEF effectively treat metastatic melanoma as a systemic disease? This question has begun to be addressed in an experimental protocol, not with B16f10 melanoma cells, but with Hepa 1-6 hepatocellular carcinoma (HCC) cells [Chen and Beebe, unpublished]. When Hepa 1-6 tumors were treated with 900 pulses at 100ns and 55kV/cm, tumors were eliminated in 6 of the 8 mice, while all control mice were humanely euthanized due to tumor burden 14-18 days after tumor initiated. When the 6 successfully treated mice were tumor free for 60 days, tumors cells were injected in the opposite flank as before. None of these animals grew tumors for 49 days before the experiment was terminated. In naïve age-match control HCC tumors grew to treatable sizes in less than two weeks. These results suggest that nsPEF ablation allows a host cell immune response. While these studies must be repeated and the mechanisms of this resistance further investigated, these results suggest that nsPEF ablation addresses another cancer hallmark, evasion of immune surveillance.

It is generally accepted that an ultimate outcome of apoptosis is the removal of aberrant cells without inflammation. This is certainly true for during development and the clearance of

immune cells, but it is likely not completely true for cancer therapeutic agents that induce apoptosis in tumors. The tumor masses are likely not cleared before some tumor cells are functionally dead. Many chemotherapeutic agents, as well as nsPEF ablation, induce cell death by apoptosis. However, it is generally considered, but not universally, that apoptosis is immunologically silent. Most chemotherapeutic agents, many of which induce apoptosis, are immunosuppressive. Conversely, considering the challenge experiments of HCC tumors presented above, nsPEF ablation may not be immunologically silent or immunosuppressive. Thus, it is possible for immune cells to present antigens from apoptotic cells. Alberts et al., [1998] demonstrated that human dendritic cells efficiently present antigen derived from apoptotic cells that stimulated class I-restricted CD8⁺ cytotoxic T-cells. When Chattergoon and co-workers [2000] engineered Fas-mediated apoptotic death of antigen-bearing cells *in vivo* by co-expressing the immunogen and Fas in the same cells, they observed that the death of antigen-bearing cells resulted in increased antigen acquisition by antigen presenting cells including dendritic cells (DCs). Casares et al., [2005] demonstrated that caspase inhibition did not inhibit doxorubicin (DX)-induced cell death, yet suppressed the immunogenicity of dying tumor cells in several rodent models of neoplasia. Further, depletion of DCs or CD8⁺T cells abolished the immune response against DX-treated apoptotic tumor cells *in vivo*. Russo et al., [2000] showed that irradiated vector-producing cells undergoing apoptosis were phagocytosed by dendritic cells (DCs). They then took lymphocytes obtained from a patient affected by a MAGE-3(+) melanoma, stimulated them *in vitro* with autologous DCs previously exposed to irradiated MAGE-3-expressing cells, which led to induction of MAGE-3-specific cytotoxic effectors, directed against a yet unknown MAGE-3 epitope. These results indicate not only that apoptotic cells and perhaps the presence of active caspases can be immunogenic, it is suggested that they may have immunogenicity. Finally, these results not only indicate that apoptotic cells can stimulate anti-neoplastic immune responses, but that they could generate cancer vaccines, having important implication for gene therapy for melanoma and other cancers.

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Part 5

Photodynamic Therapy

Can Photodynamic Therapy Be an Alternative Method in Melanoma Treatment?

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1. Introduction

Photodynamic therapy (PDT) is an effective cancer treatment that is an alternative to local therapy including surgical treatment and radiotherapy or systemic treatment such as chemotherapy or some kinds of immunotherapy. In many patients with cancer these therapeutic methods are not efficient or put their life at serious risk.

The principle of PDT is based on a photochemical process involving the absorption of light by a photosensitizer and the subsequent generation of reactive oxygen species (ROS) that induces cytotoxicity.

In the body, photosensitizers accumulate particularly in cancer cells. The precise reason for this preferential uptake or retention by malignant tissue is still unclear, but properties that appear to be involved include activated cellular uptake by neoplastic cells, differences in proliferation rates between neoplastic and normal cells, leaky vasculature within neoplastic tissue, and variations in mitochondrial potential. Moreover, PDT is a selective treatment because light delivery can be restricted to specific regions, thereby limiting activation of the drug to cured areas. These conditions mean that PDT is a safe treatment in comparison with other local treatments such as ionizing radiation and thermal destruction of tissue.

Oxygen-dependent photodynamic reactions were first described by Oscar Raab (Allison, 2010). He noted that an acridine solution killed paramecia upon irradiation with light. There was no such effect observed in the absence of light. On the basis of this knowledge about photodynamic effects, H. Tappeiner and H. Jesionek performed the first PDT sessions on a patient with skin carcinoma, with eosin as a photosensitizer (Allison, 2010). Since that time PDT has made considerable progress; new photosensitizers have been discovered and the procedure of therapy has been established.

1.1 Mechanism of action

The procedure of PDT requires two steps: administration of a photosensitizer, and its activation by light in the presence of oxygen in the tissue. Photosensitizers used in PDT should be nontoxic in their native state, but after excitation they become cytotoxic and produce local tissue destruction through intercellular generation of ROS. The light induces excitation of the photosensitizer molecules to the singlet excited state, which can be converted into the triplet excited state. The triplet state may react with surrounding

molecules in two types of photo-oxidative reaction. Type I involves electron or hydrogen atom transfer, producing radical forms of the substrate which may react with oxygen to form peroxides, superoxide ions, and hydroxyl radicals. Type II leads to the generation of singlet molecular oxygen ($^1\text{O}_2$). Both mechanisms may occur simultaneously (Lukšienė, 2003). It is known that $^1\text{O}_2$ can diffuse only 20 nm during its lifetime (Peng, 1996); hence the primary damage is closely related to the site of its formation. Therefore, cellular structures having both a high sensitizer and a high oxygen concentration will be preferentially damaged upon illumination.

In PDT also occurs a third type of reaction. In contrast to the other two it is independent on the dissolved oxygen in the tissue. In contrast to the other two it is independent of the dissolved oxygen in the tissue. The photosensitizer in the singlet excited state can react directly with biomolecules, inducing a process of photoadduct formation (Capella et al., 2003). However, this mechanism is often ignored because the electron transfer between a biomolecule and photosensitizer, and formation of free radicals, is not effective (Ochneser 1997).

1.2 Photosensitizers in PDT

The main features of an efficient photosensitizer are: low toxicity at therapeutic doses in darkness, highly tumor-targeting accumulation, homogeneous composition and stability, and absorption peaks in the low-loss transmission window of biological tissues (the far-red and near-infrared regions). An effective photosensitizer is characterized by a high quantum yield of singlet oxygen generation and/or electron transfer to substrate molecules (Granville, 2001, Lukšienė, 2003). Good photosensitizer qualities are high solubility in water, availability, uncomplicated manufacture and synthesis.

Most photosensitizers applied in PDT are based on porphyrins. The most common photosensitizers are haematoporphyrin derivatives (HPD). Its purified version named Photofrin, has an absorption peak in the red wavelength region at about 630 nm. The first compound in the porphyrin synthesis pathway (5-aminolevulinic acid, 5-ALA) is also used in PDT. 5-ALA is converted *in vivo* into the photosensitizing compound protoporphyrin IX. The second generation of photosensitizers includes compounds from other chemical groups. Most of them are cyclic tetrapyrroles consisting of substituted derivatives of porphyrin, chlorin and bacteriochlorin. Many research centers are developing new and quite different molecular structures that can act as the ideal photosensitizer (Granville, 2001).

1.2.1 Light

The photodynamic reaction is dependent on the delivery of light of an appropriate wavelength to activate a photosensitizer. The light used for photosensitization is typically in the visible or near-infrared light range. In general, with increasing wavelength in the visible and near-infrared range the depth of penetration of light increases and the absorption by hemoglobin and other endogenous chromophores decreases (Smith, 2002).

Application in PDT of higher wavelengths of light enables treatment of heavily pigmented tissues (Woodburn, 1998). Good light sources in PDT are metal halogen lamps, which emit from 600 to 800 nm radiation at high power density, and short-arc xenon lamps, tunable over a bandwidth between 400 and 1200 nm (Lukšienė, 2003).

The popularization of laser technology with optical fibers facilitates photosensitization in clinical treatment. Lasers enable the precise endoscopic application of light to almost every

site of the human body and allow the exact selection of wavelengths. Pulsed lasers, such as the gold vapor laser (GVL) and the copper vapor laser-pumped dye laser, produce brief light pulses of millisecond to nanosecond duration (Fisher 1995). Tunable solid-state lasers, such as the neodymium: YAG laser, are particularly useful for PDT. Portable semiconductor diode lasers, such as the gallium-aluminum-arsenide laser, produce light in the range from 770 to 850 nm, which corresponds to the absorption peaks of many new photosensitizers (Lukšienė, 2003).

2. Cell damage after PDT

The antitumor activity of PDT is based on four mechanisms: direct cytotoxicity, which is an intermediate reaction of singlet oxygen production with subsequent oxidation of the substrate, occlusion of blood vessels and lymphatics, and the impact on the immune response and inflammation of the body (Krawczyk-Krupka et al., 2001, Sieron & Adamek, 1999, Mazur et al., 2010). Generated reactive oxygen species (ROS) cause oxidative stress in the cells (Dellinger, 1996). The oxidative stress damage is targeted mainly at cellular macromolecules, such as lipids, nucleic acids and proteins. Hydrophobic photosensitizers accumulate mainly in cell membranes and they are primarily attacked by free radicals.

2.1 Oxidative stress

Oxidative stress is an imbalance between the formation of reactive oxygen species (ROS) and antioxidant mechanisms. Free radicals in living organisms may result from the action of endogenous factors (e.g. respiratory chain, activated leukocytes, inflammation, enzymes) or exogenous (e.g. cigarette smoke, alcohol, intense physical activity, UV radiation, ionizing radiation, car exhaust, contaminated air, physical and psychological stress). In physiological conditions the release of free radicals is controlled by the body's defense mechanisms (Sheu et al., 2006). Free radicals play a crucial role in cell functions including proliferation, apoptosis, inactivation of the growth of bacteria and viruses, intracellular signaling and intercellular communication. ROS have effects that augment or shrink the blood vessel walls, and stimulate glucose transport into cells. Disorder of the defense mechanisms under the influence of external factors or disease causes an increase in free radicals in the body, and consequently the occurrence of pathological reactions leading to damage to cells and tissues (Galecka et al., 2008, Łagowska-Lenart et al., 2008). In vitro studies show that the destructive action of free radicals may include all biomolecules in the body, causing chemical modifications and damage to proteins (denaturation), lipids (peroxidation), carbohydrates and nucleotides, resulting in changes in the structure of DNA leading to mutations (Sheu et al., 2006, Bailey et al., 2005). The concentration of oxidation products (e.g. carbonyl groups) reflects the intensity of the reaction involving reactive oxygen species. An antioxidant barrier protects the organism from the damaging effects of reactive oxygen species.

2.2 Lipid peroxidation

Lipid peroxidation (LPO) is a free radical process of oxidation of polyunsaturated fatty acids or other lipids. Lipid peroxidation proceeds in three major steps: initiation, propagation, and termination. During the first stage a fatty acid radical is produced. The hydroxyl radical is identified as initiating lipid peroxidation by abstracting hydrogen atoms from fatty-acid side chains. Cell membranes contain unsaturated fatty acids that are readily attacked by

reactive oxygen species. LPO can be initiated by hydroxyl radical ($\bullet\text{OH}$), superoxide radical ($\text{LOO}\bullet$), alkoxy radical ($\text{LO}\bullet$), alkyl radical ($\text{L}\bullet$), as well as ozone (O_3), nitrogen monoxide and dioxide, sulfur dioxides, and hypochlorite (Shevchuk et al., 2002, Bartosz, 2003).

During the propagation phase, free alkyl radicals ($\text{L}\bullet$) react with molecular oxygen to form peroxy free radicals ($\text{LOO}\bullet$). They may also detach hydrogen atoms from subsequent molecules of polyunsaturated fatty acids (LH). This cycle continues, as the new fatty acid radical reacts exactly in the same way until the termination reaction. The stage of termination occurs when a radical reacts with a non-radical. It is a "chain reaction mechanism" because a new radical is always produced. The radical reaction stops when there is such a high concentration of radical species that it can cause the impact of two radicals, forming a non-radical.

The final products of lipid peroxidation are hydroxy aldehydes (e.g. 4-hydroxynonenal), hydrocarbons (e.g. ethane and pentane) and aldehydes (e.g. malonic dialdehyde - MDA) - an endogenous genotoxic compound (Bartosz, 2003, Kessel et al., 1997, Hsieh et al., 2003). Increased production of reactive oxygen species induces an increase of lipid peroxidation products. These LPO products are able to modify the physical properties of cell membranes, leading to a loss of integrity of intracellular membranes by depolarization, increased permeability to H^+ ions, disruption and inhibition of the activity of the asymmetry of membrane enzymes and transport proteins (Hsieh et al., 2003).

2.3 Protein degradation under the influence of oxidative stress

Oxidative changes are an inherent effect of cellular metabolism and can not be eliminated. Accumulation of oxidized protein products impairs cell functions and can even induce cell death (Plaetzer 2003). ROS can induce oxidation of protein thiol groups ($-\text{SH}$) and form thiol radicals ($\text{S}\bullet$). The most degenerating for the cell during oxidative stress is oxidation of thiols in the membrane. It can lead to membrane disintegration and increased permeability. Oxidative damage of $-\text{SH}$ groups causes rapid loss of biological activity of protein. These radicals in turn oxidize to disulfides, forming intermolecular disulfide bridges. These reactions cause irreversible changes in membrane structures, which are dangerous to the life of the cell (Wang 2001).

2.4 Changes in DNA

DNA encodes genetic information, so any changes that occur in the genome are extremely dangerous for the cell. The number of daily incidents of DNA damage exceeds 10 000; therefore there are corrective mechanisms for sensing and correcting the damage. The mechanisms of DNA damage induced by photodynamic therapy are not well known. PDT can cause cross-linking of DNA strands (McNair et al., 1997, Haylett et al., 2003, Woods et al., 2004). DNA damage caused by photodynamic therapy may occur in cancer cells and normal cells surrounding the tumor. PDT generates through the photosensitizer reactive oxygen species. Singlet oxygen has a very limited range ($< 0.1 \mu\text{m}$) and life-span (less than 1 s). The probability of DNA damage is low, unless it is generated in the close proximity of a DNA strand. Current studies suggest that cell death after PDT is caused by damage to cytoplasmic proteins and mitochondria, and not damage to DNA (Miller et al., 2007). Normal cells are able to cope with the damage after PDT but this is dependent on the type of photosensitizer (Woods et al., 2004).

2.5 Antioxidative system

To protect against free radical damage the body uses a number of mechanisms to provide antioxidant protection of the body. Structural cell organization is one of these mechanisms. This specific formation enables isolation of those places where free radical reactions take place (Halliwell, 2000, Huang, 2000). In addition various metabolic mechanisms play an important role in protection against free radicals. The knowledge of oxidative damage caused by PDT is important in developing strategies for treating tumours and the potential reduction of side effects. They have been divided into the following categories (Santiard, 1995):

- Reactions with suppressive compounds (carotenoids, vitamin E);
- Non-enzymatic mechanisms: antioxidants, free radical scavengers, ions of transition metals, sequestration of metals, metallothioneins;
- Enzymatic mechanisms: superoxide dismutase, catalases, heme oxygenase, glutathione peroxidase, glutathione reductase, glutathione S-transferase and groups of secretory phospholipases A₂;
- Reactions with heat shock proteins – proteases and chaperone proteins.

2.5.1 MnSOD

MnSOD is a mitochondrial form with Mn in the active center. Human MnSOD is a tetrameric enzyme with four identical subunits each harboring a Mn⁺³ atom (McCord, 2002). It was demonstrated that transient transfection with the MnSOD gene resulted in decreased effectiveness of PDT. These results suggest that MnSOD plays a leading role in the response to PDT and that abnormal mitochondria may be a decisive factor in phototoxicity (Saczko et al., 2007). MnSOD is a critical antioxidant enzyme residing in mitochondria. Golab et al. observed that following PDT there was induction of MnSOD expression in tumor cells (Golab et al., 2003). The group of SODs is emerging as significant antioxidative enzymes that can regulate the sensitivity of cancer cells to different treatment modalities. Some authors have also observed that overexpression of MnSOD suppresses apoptosis (Kuroda et al., 2000).

2.5.2 Heme oxygenase-1 (HO-1)

Heme oxygenase exists in two isoforms: inducible (HO-1) and constitutively expressed (HO-2). Heme oxygenase-1 (HO-1) is an enzyme that catalyzes the degradation of heme to Fe²⁺ ion, carbon monoxide and biliverdin, and then biliverdin is converted to bilirubin by biliverdin reductase (Dulak & Jozkowicz, 2003). Biliverdin and bilirubin are antioxidants, and may therefore play a very important role in cytoprotection of cells exposed to the oxidative stress induced by PDT. Induction of HO-1 by PDT was described in the CHO (Chinese hamster ovary) cell line using Photofrin (Gomer, 1991). The photodynamic reaction induces HO-1 expression. HO-1 also plays a protective role in cells where PDT was applied. The removal of iron ions with desferrioxamine from cells after PDT resulted in increased cell death, which confirms the key role of ferrous ion generation in HO-1 mediated cytoprotection (Nowis et al., 2006).

2.5.3 Heat shock proteins

The heat shock proteins (HSPs) are a class of proteins whose expression is increased when cells are exposed to different kinds of stress such as heat shock, pH shift, hypoxia, osmotic

and oxidative stress. Production of HSPs may also grow in response to infection, inflammation, effects of toxins, UV radiation, starvation, etc. HSPs work as chaperone proteins responsible for the proper folding of other proteins, translocation and degradation. They also participate in protein assembly, export, turnover and regulation. HSPs are found in virtually all living organisms. In normal conditions cellular levels of HSPs are stable and low, but if cells are exposed to any kind of stress, the levels of HSP quickly increase (Pockley, 2003). HSPs are generally classified according to their molecular weight, which can vary from 10 to 170 kDa, e.g. HSP70 (Hightower & Hendershot, 1997). PDT induces different HSPs including HSP27, HSP60, HSP70, HSP90, GRP78 and GRP94. A protective role in PDT-treated cells has been shown, for example survival of tumor cells exposed to PDT induced overexpression of HSP27 (Wang et al., 2002). When there are damaged proteins, HSP binds damaged molecules. This results in dissociation of HSF (heat shock factor), then migrates to the nucleus, where it binds with HSE (heat shock elements) leading to HSP overexpression (Morimoto, 1993). HSP90 inhibits formation of an active apoptosome whereas HSP70 prevents the recruitment of procaspase-9 to the apoptosome complex (Almeida et al., 2004).

2.5.4 GST π

Glutathione S-transferases (GSTs) are crucial in protection of cells against oxidation products. There are enzymes of the second phase of metabolism that counteract the process of carcinogenesis (L'Ecuyer 2004). This group of enzymes plays a key role in detoxification and reduction of reactive oxygen forms, especially the isoform GST- π . This enzyme protects cells against DNA disintegration and drug toxicity. Genetic polymorphisms in glutathione S-transferase and its altered expression and activity are associated with oxidative DNA damage which in turn leads to increased susceptibility to cancer (L'Ecuyer et al., 2004). A high level of GST- π expression is related to the development of drug resistance in cancer cells not only by increased detoxification of anticancer agents, but also by suppression of cellular ROS which induce cell death. This means that a high level of GST- π may be a prognostic factor in malignant diseases (Aliya et al., 2003).

2.5.5 Small molecule antioxidants

Molecular reactions of antioxidants with ROS are less specific than the effect of antioxidant enzymes, which means that these compounds are more versatile defenders. The body's antioxidant status is also dependent on the level of exogenous antioxidants, mainly supplied with food. An important role is played by vitamin E. Because of its lipophilic properties it may have a protective effect in relation to membrane phospholipids, protecting them from peroxidation. One free radical could trigger the whole cascade of reactions leading to oxidation of unsaturated fatty acids. This cascade proceeds until another formed fatty acid radical meets on their way vitamin E, which breaks the chain reaction. Reduced vitamin E can be regenerated by vitamin C that has the ability to stabilize the hydroxyl radical (Chow et al., 1999, Rahman, 2007, Sies et al., 1992). Vitamin C reacts with the hydroxyl radical and singlet oxygen. Vitamin C with glutathione coenzyme Q, cysteine, uric acid and bilirubin are among the active antioxidants in the aqueous phase. Vitamin C inhibits the peroxidation of hemoglobin and with coenzyme Q and reduced glutathione protects mitochondria from oxidative damage (Kurl et al., 2002). The effect of vitamin E supplements β -carotene and its metabolite, vitamin A. The precursors of vitamin A include carotenoids, the main dietary source of vitamin A in humans. In the gastrointestinal tract arises retinal, which is then converted to retinol (Rahman, 2007, Sies et al., 1992).

3. Mechanism of cell death

Oxidative stress is a factor which initiates cell death after photodynamic reaction (Almmeida et al., 2004, Castano et al., 2005, Kulbacka et al., 2010, Saczko et al., 2009). In this part of our review we will examine the mechanisms of cell death caused by ROS and related metabolites, which are stimulated during the photodynamic process. The type of cell death by PDT may be mainly apoptosis or necrosis depending on the properties and concentration of the photosensitizer connected with the irradiation dose. However, another mode of cell death was also observed after photodynamic treatment, i.e. autophagic. This type II cell death is characterized by an enormous increase of two-membrane autophagic vacuoles in the cytoplasm which are finally catalyzed by lysosomal hydrolases (Edinger et al., 2004, Kroemer et al., 2005, Golstein et al., 2006). Autophagy is a convertible process, which can provoke both survival and death pathways, in contrast to the apoptotic irreversible process leading only to cell death (Kessel et al., 2007). Apoptosis (type I cell death) is genetically and morphologically different from necrosis. Apoptosis represents a regulated, universal and perfectly efficient, strongly controlled energetic suicide pathway. This process requires the activation of some hydrolytic enzymes – proteases and nucleases – leading to DNA fragmentation and destruction of cell structures. The morphological changes connected with apoptosis include nuclear pyknosis, DNA fragmentation, membrane blebbing, cell shrinkage and production of apoptotic bodies which are removed by adjacent cells and resident phagocytes (Kroemer et al., 1998 and 2005, Ryter et al., 2007, Kroemer et al., 2007). Apoptotic cell death is the most preferable effect of various anticancer therapies which leads to destruction and elimination of pathological cells. Inflammation does not occur through apoptosis in cancer cells and surroundings tissue (Fiers et al., 1999). On the other hand, necrosis (type III cell death) appears in a negative way as death associated with gross membrane damage and cell outflow into the extracellular space. This characteristic modification may lead to local inflammation and injury of nearby tissue. The endpoint of necrosis is manifested in cell swelling or oncosis (Majno et al., 1995). However, a recent study showed that necrosis can also occur similarly to programmed cell death, which consists of induction, commitment and execution processes of necrosis triggering the cysteine cathepsin-mediated lysosomal death pathway (Leist et al., 2001, Bizik et al., 2004, Golstein et al., 2005, Golstein et al., 2006). In general, it can be observed that lower doses of PDT lead to apoptosis, while higher doses lead to necrosis in cells (Ketabchi et al., 1998, Kessel et al., 2000). A previous study showed that the three well-known types of cell death are dependent on the developmental stage, physiological conditions of cells and the nature of the death signal (Majno 1995). Mixed-type cell death forms, containing characteristic properties for both types, apoptosis and necrosis, have been noted (Wang et al., 2003).

3.1 Apoptosis following PDT

The photodynamic reaction activates different signal transduction pathways connected with transcription factors and cell cycle regulation, which often lead to cell death or survival (Pazos et al., 2007, Robertson et al., 2009). The different types of cell death-induced photodynamic processes are permanently under investigation. However, the majority of studies have shown that apoptosis is a rapid and dominant type of cell death following the photodynamic reaction, in different experimental conditions and a variety of sensitizers (Oleinick et al., 2001). On the other hand, cancer cells can die in other ways: necrosis or autophagy (Buytaret et al., 2006). Autophagic and programmed necrosis are rarely related to

PDT. There are a few commonly known significant factors which can induce necrosis: extra-mitochondrial location of photosensitizers, a high dose of PDT, and glucose starvation (Almeida et al., 2004, Kieslich et al., 2005, Nowis et al., 2005). Also the cells' sensitivity to PDT conditions and their genotype may control the type of cell death following PDT (Wyld et al., 2001, Bar et al., 2007, Saczko et al., 2005). The most significant factors determining the effect of PDT are the physicochemical properties, intracellular distribution of photosensitizers, and the interactions between different cellular organelles such as mitochondria, lysosomes, endoplasmic reticulum (ER), Golgi apparatus and plasma membranes. The uptake of photosensitizers into cancer cells plays a decisive function in increasing the efficiency of PDT (Pazos et al., 2007, Robertson et al., 2009). Numerous studies with various first and second generation photosensitizers have concluded that the induction of apoptotic cell death by PDT was connected with mitochondrial damage (Kessel et al., 1998, Saczko et al., 2005 and 2007, Chwiłkowska et al., 2006). Kessel and other researchers have demonstrated that sensitizers which localize in mitochondria are much more rapid inducers of apoptosis than photosensitizers localized in endoplasmic reticulum, Golgi apparatus, lysosomes and plasma membranes (Kessel et al., 1997, Dahle et al., 1990, Marchal et al., 2004). On the other hand, cationic photosensitizers or others accumulating in plasma membrane were associated with membrane photodamage and necrotic results (Kessel 1998, Luo 1996). Moreover, the availability of oxygen, the suitable wavelength and intensity of light, as well as the biological conditions and type of cancer cells can influence the mode of cell death (Rosa et al., 2000, Castano et al., 2004, Juzeniene et al., 2007, Plaetzer et al., 2003). Photodynamic therapy can induce apoptosis in two dissimilar pathways: intrinsic, mitochondria-dependent, and extrinsic, death receptor-dependent (Almeida et al., 2004).

3.2 Intrinsic pathway

Mitochondria play a crucial role in intrinsic pathways of apoptotic cell death, due to mitochondrial damage being the major object of photocytotoxicity. The mitochondrial pathway of apoptosis is often mediated when sensitizers are accumulated in these organelles, but it is not obligatory. The loss of mitochondrial transmembrane potential and the release of cytochrome c from the mitochondrial inner membrane space to the cytosol is a well-examined apoptotic occurrence, which has also been documented for numerous photosensitizers localized in mitochondria such as Photofrin, benzoporphin derivative, cyanine and phthalocyanine (Fabris et al., 2001, Pervaiz et al., 1999, Saczko et al., 2007). The present observation of apoptosis by the mitochondrial pathway shows that cytochrome c released to the cytoplasm forms a triple complex with the apoptosis-inducing factor (Apaf1) and procaspase 9, which leads to activation of procaspase 9 and following activation of executioner caspases. Uncontrolled generation of reactive oxygen species during PDT can lead to endoplasmic reticulum stress. It results in perturbation of the Ca^{2+} gradient, which can lead to the creation of a permeability transition complex and the involvement of Bcl-2 proteins (Grebenova et al., 2003, Buytarek et al., 2006, Nowis et al., 2005). Some data show that the endoplasmic reticulum as well as mitochondria may induce apoptosis and autophagy in cancer cells following the photodynamic reaction. Manipulation of the PDT conditions results in suppression of apoptosis with increasing autophagy in mouse L1210 leukemia cells (Kessel et al., 2007). The hydrophilic chlorine photosensitizers localize mainly in lysosomes and activate the mitochondrial apoptotic pathway following photodamage of lysosomes. The lysosomal proteases are released by lysosomal injury during the photodynamic reaction and activate caspases directly or indirectly through mitochondrial

damage. However, the mechanisms by which lysosome-localizing sensitizers induce mitochondrial apoptotic cell death are not fully elucidated (Nakajima et al., 1992, Mori et al., 2000, Nagata et al., 2003, Reiners et al., 2002).

3.3 Activation of caspases

The apoptosis effectors contain intracellular proteases termed caspases. Caspases are a family of intracellular endopeptidases that are synthesized as zymogens and are converted into active enzymes. Caspases can be divided into two groups: initiators (numbers 1, 2, 4, 5, 8, 9, 10 and 12) and effectors (numbers 3, 6 and 7) (Castano et al., 2005). The properties of apoptotic cell death are dependent on proteolytic cleavage of cellular substrates by effector caspases and can be activated by three dissimilar pathways involving intrinsic apoptosis activated by caspase 9, an extrinsic way connected with caspase 8, and an endoplasmic reticulum stress one induced by caspase 12.

The mitochondrial pathway is associated with release of cytochrome c proteins by mitochondria into the cytoplasm involving establishment of caspase 9 and cleavage of executioner caspases 3, 6 or 7 (Ketabchi et al., 1998, Robertson et al., 2007). This leads to degradation of various proteins and DNA and finally cell death by apoptosis. Some investigations suggest that high doses of photodynamic therapy can photochemically inactivate crucial enzymes and other components of the apoptotic cascade such as caspases (Lavie et al., 1999). A previous study examined the level of expression of caspase 3, 8 and 9 after photodynamic reaction with Photofrin® (Ph) using immunocytochemical assay (ABC method) in MCF-7 (human breast adenocarcinoma cell line) and A549 (human lung carcinoma cells). Differentiated expression of caspases 3, 8 and 9 in A549 cells was observed. The intensity of immunocytochemical staining of caspases depends on the concentration of Photofrin®, the time of irradiation and the incubation time after irradiation. Detection of two initiators, caspases 8 and 9, after photodynamic reaction in human lung adenocarcinoma A549 cells may suggest that apoptosis activation occurs by two independent signal transduction pathways, which are relative (Fabris et al., 2001, Grebenova et al., 2003, Saczko et al., 2007). Also a mechanism has been documented in which caspase 8 cleaves Bid protein and there follows mitochondrial release of cytochrome c dependent activation of caspase 9 (Buggiani et al., 2008). Moreover, a variety of apoptosis activation pathways induced during the photodynamic reaction can result in a lot of alternatives and cross-link the apoptotic signal transduction pathway (Almeida et al., 2004, Mitsunga et al., 2007).

3.4 Bcl-2 family proteins and p53 status

The Bcl-2 family proteins are divided into two groups: antiapoptotic Bcl-2, Bcl-X_l, and Bcl-w, and proapoptotic Bax, Bak, Bad, and Bim, which respectively inhibit or support the execution of apoptotic cell death. Thus families of proteins control mitochondrial stability by maintaining the balance between proapoptotic proteins that translocate to the mitochondria and antiapoptotic ones that exist in the mitochondrial membrane (Ryter et al., 2007, Kelekar et al., 1998). The *Bcl-2* gene product is located in the membranes of the endoplasmic reticulum, nuclear envelope and the external membranes of the mitochondria (Ryter et al., 2007). The most significant objective of PDT in the cell signaling pathway is activation of the proapoptotic proteins belonging to the Bcl-2 family. Photodamage after photodynamic reaction provokes activation of these proteins and induces apoptosis in malignant cells. Bax protein expression has been linked to a beneficial reaction to PDT. It can be used to assess

the cancer response to photodynamic reaction (Zhen-hui Peng et al., 2008, Roland et al., 2007, Robertson et al., 2007). Generally the role of Bax protein in the release of cytochrome c from mitochondria into the cytoplasm and activation of the apoptotic process has been established in many systems. Chiu and co-workers observed that phthalocyanine Pc4-PDT induced Bax dislocation from the cytoplasm to mitochondria as early signaling for the mitochondrial pathway of apoptosis in human breast cancer MCF-7c3 cells (Chiu et al., 2003). Phthalocyanine Pc4 is a new generation sensitizer that localizes in intracellular membranes, particularly in mitochondria. Pc4-PDT photodamages antiapoptotic Bcl-2 and Bcl-xL proteins and activates apoptosis in cancer cells (Castano et al., 2005, Kessel et al., 2000, Xue et al., 2001). Usada et al. examined the cells with transfection and wild-type Bcl-2. Overexpression of Bcl-2 decreased apoptosis, which is connected with inhibition of the proapoptotic Bax protein. On the other hand, high doses of Pc4-PDT are necessary to activate Bax in cells with increasing expression of antiapoptotic Bcl-2 protein (Usada et al., 2003). The p53 tumor suppressor gene takes part in the response to DNA damage, connected with cell cycle regulation, DNA repair, and induction of apoptosis (el-Deiry et al., 1998, Bunz et al., 1999). Expression of wild-type p53 activated by chemotherapy, radiation or photodynamic reaction increases the sensitivity to apoptosis, whereas mutated p53 decreases the sensitivity. In many cases of cancer, mutated p53 was found (Lowe et al., 1994). P53 plays a crucial role in regulation of proapoptotic Bcl-2 proteins. Bax induced the mitochondrial pathway by outflow of apoptogenic proteins, such as cytochrome c. However, in different studies the involvement of Bax and p53 in PDT-mediated apoptosis was observed (Fisher et al., 1997, Fisher et al., 1998, Zhang et al., 1999). Some data indicate that p53 is required for caspase 3 activation, suggesting that p53 may play a role in PDT-activated early apoptosis in cancer cells (Mitsunga et al., 2007). Bar and co-workers examined the effect of Photofrin-PDT (Ph-PDT) on clear human ovarian carcinoma with a "silent" mutation in the p53 gene. The cells were dying in a necrotic way. They postulated that this mutation can inhibit apoptosis in these cells (Bar et al., 2007). The modification of Ph-PDT by 2-methoxyestradiol leads to activation of apoptotic cell death in these cells (unpublished data). Another study showed that PDT induced apoptosis in cancer cells independent on p53 (Almeida et al., 2004).

3.5 Extrinsic apoptosis pathway

This pathway is connected with initiation of the apoptotic process in response to induction of a cell surface receptor, such as the tumor-necrosis factor receptor (TNF-R) family of death receptor, or the apoptotic program can be initiated when a death ligand (Fas ligand – FasL) interacts with a cell surface receptor (Fas/APO-1/CD95) (Mupidi 2004). Death receptor mediated apoptosis occurs during photodynamic therapy when photosensitizers preferentially localize in the cell membrane. In human epidermoid carcinoma A431 treated photodynamic reaction with phthalocyanine 4 increasing levels of the surface death receptor Fas and of its ligand FasL were observed. Additionally, in the same conditions caspase-8 cleavage was increased in Pc4-sensitized A431 cells. The function of the Fas/FasL system in PDT was also observed in canine kidney cells and hamster lung fibroblast after photosensitization with Photofrin (Almeida 2004). PDT-induced apoptosis is dependent on many metabolic signals which have an influence on the apoptotic pathway. Many examinations suggest that both the intrinsic and extrinsic pathway were crosslinked during photosensitization.

4. PDT in melanoma treatment

Results from preclinical and clinical studies conducted worldwide over a 25-year period have established PDT as a useful treatment approach for some cancers (Mitton D, 2005). For more than 20 years scientists have maintained that PDT is not effective for pigmented melanomas (Ambrosone et al., 2005). Scientists focused on the improvement of inefficient PDT due to the competition between the absorbance of melanin from melanoma and the absorbance of photosensitizers at the photosensitizer excitation light wavelength. Melanin absorbs light over the entire wavelength region used for PDT (400-750 nm) (Ma et al., 2007). This was one reason why pigmented melanoma has been excluded in some PDT studies (Lim et al., 2004). It is well known that the longer wavelength of light absorption is, the deeper photosensitizer penetrates the skin (Woodburn et al., 1998). Since establishing the substantial heterogeneity of melanomas and their varying resistance to radio- and phototherapy, there have been intensive studies on the molecular processes occurring in these neoplasms during therapy (Kusmierz et al., 2009).

The limitations of the porphyrin-derived photosensitizers with regard to light penetration into tissues promoted the synthesis of longer wavelength absorbing photosensitizers (Kreimer- Birnbaum, 1989). PDT treatment with lutetium texaphyrin (PCI-0123) (excitation at 732 nm) promoted survival of mice bearing the highly pigmented B16F10 melanoma (Woodburn et al., 1998). Also in these studies tumor apoptosis was evident in the PDT-treated neoplasms after irradiation. PDT is a process with membranes being a principal target (Henderson et al., 1992). Woodburn et al. performed a confocal laser scanning microscopy studies with PDT and PCI-0123. There was proved that PDT destroys the melanosomal membrane, which leads to death due to oxidative stress of cells (Woodburn et al., 1997 and 1998).

An important aspect of the antitumour effectiveness of PDT is related to the distribution of the photosensitizer. The localization of the drug in cytoplasmic organelles during PDT plays a major role in cell death (Saczko et al., 2007). It was suggested that localization of photosensitizing drugs in the plasma membrane, or in lysosomes, leads to necrosis after PDT treatment. Localization in the mitochondrial membrane causes apoptosis (Chen et al., 2000). Intracellular accumulation of the photosensitizing drug is one of the most important factors to determine the efficacy of PDT (Saczko et al., 2007; Lam et al., 2001; Ogura et al., 2003). Photofrin (Ph) was the first photosensitizer used in PDT of cancer. This drug has proved to be effective in the treatment of many cancers (Almeida et al., 2004). Ph has been involved in several mechanisms in PDT, such as cytotoxicity and apoptosis (Konan et al., 2002). Saczko et al. showed that the intracellular localization of Ph in a cultured malignant melanoma (Me45) cell line is mainly intracellular compartments and mitochondrial membranes (Saczko et al., 2007). Ricchelli et al. also reported that Ph was localized in the mitochondria *in vitro* (Ricchelli et al., 1990). Mitochondria play an important role in the early events of apoptosis (Lam et al., 2001; Patito et al., 2001). Marchetti et al. demonstrated that Ph is a ligand for the mitochondrial peripheral benzodiazepine receptor, which is responsible for triggering pore transition (Saczko et al., 2007, Marchetti et al., 1996).

The mitochondrial apoptotic pathway was also shown by Chen et al. in therapy with methylene blue (MB) (Chen et al., 2008). It is a photosensitizer which has excellent photochemical properties. MB is well known to have a high quantum yield of intersystem crossing and singlet oxygen generation and can produce radical species in the presence of reducing agents (Gabielli et al., 2004; Tardivo et al., 2005). This photosensitizing drug has

affinity for melanocyte-produced melanin, which contributes to selective absorption of this compound by cutaneous melanomas (Link et al., 1989). MB showed enhanced penetration efficiency into melanoma cells and also in mitochondrial membranes, which induced apoptotic cell death by causing mitochondrial dysfunction (Ball et al., 1998). Chen et al. showed that MB-PDT could induce apoptotic cell death through the photochemical generation of reactive oxygen species that activate the caspase-9 and caspase-3 apoptosis pathway (Chen et al., 2008). A transplantable mouse melanoma model showed that the tumor size in treated mice decreased, which was associated with enhanced apoptotic cell death.

Nowak-Sliwinska et al. have tested several photosensitizers to evaluate their photoefficiency in the Cloudman S91/I3 mouse melanoma cell line (Nowak-Sliwinska et al., 2006). They compared the efficiency of the photodynamic effect using cyanine (MC540) and porphyrin derivative photosensitizers (Photofrin and verteporfin). Their study showed that verteporfin and Photofrin are effective compounds with the classical type II mechanism of photodynamic reaction, but verteporfin is the most potent photosensitizer against melanoma cells. Generation of singlet molecular oxygen by photo-activated verteporfin was more than twice as high as in the case of Photofrin. The LD50 light dose for verteporfin was four times lower than for Photofrin, in spite of the much lower concentration used (2 vs. 10 lg/ml) and 10 times higher for MC540 than for Photofrin. Buseti et al. described the pharmacokinetics of verteporfin in mice and showed that a single treatment inhibited growth of B16 tumor (Busetti et al., 1999).

Induction of apoptosis by PDT seemed to play an important role in the photodynamic treatment efficacy (Barge et al., 2004). Ph-PDT induces cell death in the human Beidegröm Melanoma (BM) cell line mainly through apoptosis (Saczko et al., 2005). The application of comet assay to study the influence of Ph-PDT on BM cells showed that some types of DNA damage depend on photosensitizer concentration, as well as on time and dose of irradiation. Similar results were obtained by Barge et al. (Barge et al., 2004) in human melanoma cells using a new silicon phthalocyanine photosensitizer and by Haddad et al. in murine malignant melanoma *in vitro* and *in vivo* using aluminum phthalocyanine (AlpcS4) (Haddad et al., 1998)

Higher doses of light and sensitizer lead to a dose-dependent augmentation of reaction oxygen species in melanoma cells (Kästle et al., 2011; Kolarova et al., 2007). A widely used photosensitizer is the protoporphyrin IX (PpIX) precursor 5-aminolevulinic acid (ALA). ALA is the first molecule in heme biosynthesis which is converted to the active photosensitizer PpIX in the mitochondria. Kästle et al. showed that WM451LU cells are more affected by ALA-PDT than non-tumorigenic keratinocytes (Kästle et al., 2011). The reason for this is elevated porphobilinogen deaminase activity and a significant decrease in ferrochelatase activity in cancer cells (Dailey et al., 1984; Kästle et al., 2011; Leibovici et al., 1988). What is more, PDT of melanoma with porphyrin and porphyrin derivatives is effective and well tolerated by patients (Szurko et al., 2003).

Kolarova et al. studied the effects of zinc-5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrin (ZnTPPS₄) on human G361 melanoma cell line (Kolarova et al., 2005). Analysis of DNA damage in the cell line after PDT was proved by comet assay. This treatment method gave rise to DNA damage. Further studies demonstrated that ZnTPPS₄ induces the highest ROS production in the G361 cell line compared to other porphyrins - TPPS₄ and PdTPPS₄. A correlation was observed between ROS production and cell survival. The results

demonstrate that the photodynamic effect depends on sensitizer type, its concentration and light dose (Kolarova et al., 2007). Later research showed the most significant phototoxic effect of chloraluminium phthalocyanine disulfonate (ClAlPcS₂)-PDT in spite of significantly higher ROS production induced by ZnTPPS₄-PDT on G361 cells (Krestyn et al., 2010).

HO-1 has a number of potential protective effects against oxidative stress. Additionally, the last catalytic product of HO-1 is CO. CO acts as a second messenger. It has anti-inflammatory and anti-apoptotic effects (Bilban et al., 2008). Its expression is closely related to the oxidative stress levels in the cells (Saczko et al., 2007). An increase in expression of HO-1 following photodynamic therapy was observed by Gomer et al. (1991) and Nowis et al. (2006). They demonstrated that overexpression of HO-1 protects cancer cells against PDT with Photofrin. Other authors have also reported that PDT with 5-ALA resulted in increased formation of ROS and further enhancement of HO-1 induction (Frank et al., 2007). Kästle et al. applied inhibitors of HO-1 and PARP what improved the efficiency of photodynamic treatment (Kästle et al., 2011). Drastic reduction in viability by the addition of ZnPpIX is among other things the result of higher ROS levels (75% more than without ZnPpIX) after irradiation and inhibition of HO-I (Kästle et al., 2011). Also inhibition of the protein PARP-1 seems to be a plausible addition to anticancer treatment. PARP is a protein involved in a number of cellular processes. One of its important functions is to assist in the repair of single-strand DNA breaks. If one single-strand broken DNA is reduplicated, a double-strand broken DNA results. If PARP is inhibited, cell death occurs (D'Amours et al., 1999). In the co-culture 65% of WM451LU melanoma cells and 35% of HaCaT keratinocytes were present before PDT. After PDT the proportion was 41% of melanoma cells and 59% of HaCaT cells. Combination of both inhibitors with PDT improves these results to 16% of melanoma cells and 84% of HaCaT cells. Addition of HO-1 and PARP inhibitors significantly improves the efficiency of photodynamic treatment (Kästle et al., 2011).

A very interesting study was presented by Ma et al. (2007). In that work reflectance spectroscopy was applied to study depigmentation of human and murine skin with different melanin contents, and effects induced by PDT with topical application of methyl 5-aminolevulinate (MAL) on B16F10 melanotic melanomas transplanted to mice. Skin depigmentation leads to increase in reflectance. PDT with violet light (420 nm) bleached some of the melanin in the skin above the B16F10 melanomas. It was concluded that violet light in PDT can bleach melanin in melanotic tumors and therefore increase their sensitivity to red light (634 nm). This finding indicates a new PDT modality (Ma et al., 2007).

The direct effects of PDT are cytotoxic for cancer cells, but it also could induce an immune system response, what has been proved *in vitro* (Blom et al., 1997). Treatment of ocular melanoma cells with hematoporphyrin ester (HPE)-PDT temporally alters the expression of HLA class I and β 2-microglobulin. This may affect anti-tumor-immune responses.

5. Conclusions

Photodynamic therapy, a minimally invasive therapeutic modality, has been shown to be effective in a number of oncological and non-oncological conditions. The advantages of PDT application are better cosmetic outcomes without surgical scars or the discoloration that is evident in surgery or cryotherapy. Moreover, PDT may be used to decrease the size of a large tumor, which can be subsequently removed with a smaller excision. The experience in the use of PDT in the treatment of other skin diseases, including psoriasis, sarcoidosis, acne and human papillomavirus infections, is preliminary and undergoing clinical testing.

Although the idea of PDT usage is rather simple, the procedure is complicated by several parameters such as the formulation of the photosensitizing agents, the mode of delivery and duration of application, as well as the multiple light-specific parameters, such as exact wavelength, duration and intensity of various light sources. With the application of second-generation, stable, lipophilic photosensitizers with optimized wavelengths, PDT may be a promising tool for therapy in skin cancer. Melanoma is the most severe of all skin neoplasms as it may grow rapidly and metastasize. For many years melanoma, mainly due to the presence of melanotic biopolymers and the presence of gene mutations which code pro-apoptotic proteins, has been included in the group of resistant irradiation cancers. However, the research in recent years has indicated high variation in sensitivity in melanoma cells. This information has led many researchers to focus on examining the potential use of photodynamic therapy (PDT) in the fight against this cancer. The application of photodynamic therapy opens new perspectives in treatment of this tumor. Photodynamic therapy is an effective local cancer treatment that induces cytotoxicity through intercellular generation of reactive oxygen species. There are three indispensable components in this therapy: a photosensitizer, light, and oxygen inside the diseased tissue. The photosensitizer is accumulated in the target cells and absorbs light of certain wavelength. The energy is transferred to oxygen and highly reactive oxygen species are generated. This is usually singlet oxygen. Potential cellular targets for photodynamic therapy are the cell membrane and membranous organelles such as mitochondria, lysosome and nuclei. The disintegration of cellular structures and modulation of genetic information induced by PDT leads cancer cells to a death pathway. Numerous studies suggest that the exposure of tumor cells to PDT can lead to cell death via two separate processes: apoptosis and necrosis. In contrast to necrosis, apoptosis is an energy-dependent, distinct form of cell death that follows a sequence of genetically programmed events and proceeds without inflammation. Moreover there was also noticed that photodynamic therapy can increase the radical and the oxidant processes in oncological patients whose antioxidative mechanisms are overworked. Potential targets for PDT in melanoma eradication include cell proliferation inhibition, activation of cell death, reduction in pro-survival autophagy and a decrease in the cellular melanocytic antioxidant system. As we have indicated, more research on PDT with melanoma is needed to determine the exact parameters optimal for the treatment of this disease. In our opinion PDT should be considered as a good candidate for treatment in malignant melanoma. This technique may be also a promising device for melanoma in combination with standard therapies.

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7. References

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Part 6

Clinical Trials

Update on Clinical Trials for Malignant Melanoma

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1. Introduction

After more than 20 years of little improvement in the outcome for patients with malignant melanoma despite a huge international effort spanning basic and clinical research, the last 2 years have shown significant steps forward in treatment options. This year has seen one new drug (Ipilimumab) being licensed and a second (vemurafenib) submitted for approval. This chapter focuses on the key clinical trials in the last 5 years, and gives an indication of the challenges ahead to ensure optimal use of these effective new therapies.

2. Adjuvant treatment

The only agent currently approved in the adjuvant setting for patients with completely resected malignant melanoma is interferon alfa (IFN alfa). A large number of randomised studies evaluating different treatment doses and schedules in a range of American Joint Committee on Cancer (AJCC) stages have been completed.

Despite this, there is still no consensus on the role of IFN as adjuvant treatment and reports have been conflicting in terms of therapeutic efficacy. The majority of studies compared adjuvant IFN to observation alone with a smaller number of studies using vaccines or IFN and chemotherapy as a comparator arm (Kirkwood et al. 2001). This chapter focuses on those studies that have compared interferon to observation only and these are summarised in table 1. Of the 13 published trials, 7 have demonstrated a benefit for IFN over observation in terms of disease free survival (DFS) and 2 have demonstrated an overall survival (OS) benefit (E1684 and DeCOG) (Kirkwood et al. 1996; Garbe et al. 2008)

The Scottish MG trial demonstrated benefits for both DFS and overall survival at 2 years but this diminished with longer follow up and was not statistically significant at 6 years (Cameron et al. 2001).

A meta-analysis of 12 trials was reported in 2003 including 10 trials with IFN compared to observation and 2 studies comparing IFN to a GMK vaccine (Wheatley et al. 2003). This meta-analysis demonstrated a 17% reduction in the risk of recurrence following treatment with IFN compared with control (HR 0.83, $p < 0.001$) but no benefit on overall survival (HR 0.93, $p = 0.1$).

Trial	Stage	IFN regimen	No	Results
NCCTG (Creagan et al. 1995)	IIA-III	IFN-a 20MU/m ² im x3/wk x 12wk	IFN=131 Obs=131	DFS: HR 0.83 (0.61-1.13; ns) OS: HR 0.9 (0.64-1.25; ns)
E1684 (Kirkwood et al. 1996)	IIB-III	IFN-a 20MU/m ² iv x5/wk x4wk then 10MU/ m ² sc x3/wk x48wk	IFN=147 Obs=140	5yr RFS 37% vs 26% (p=0.0023) 5yr OS 46% vs 37% (p=0.0237)
Austrian MMCG (Pehamberger et al. 1998)	II	IFN-a 3MU sc x7/wk x3wk then 3MU sc x3/wk x49wk	IFN=154 Obs=157	RFS 36% vs 24% (p=0.02) OS 13% vs 11% (ns) Median FU 3.4years
French CGM (Grob et al. 1998)	II	IFN-a 3MU sc x3/wk x18mo	IFN=253 Obs=246	DFS: HR 0.75 (0.57-0.98; p=0.035) OS: HR 0.72 (0.51-1.01; p=0.059)
E1690 (Kirkwood et al. 2000)	IIB-III	High: IFN-a 20MU/m ² iv x5/wk x4wk then 10MU/ m ² sc x2/wk x48wk Low: IFN-a 3MU/m ² sc x3/wk x24mo	IFN High=215 Low=215 Obs=212	DFS: High vs obs: HR 1.28 (1.0-1.65; p=0.05); Low vs Obs: HR 1.19 (0.93-1.53; p=0.17) OS: No differences between the 3 groups.
Scottish MG (Cameron et al. 2001)	IIA-III	IFN-a 3MU sc x3/wk x6mo	IFN=47 Obs=49	Median DFS 22 mo vs 9 mo (ns) Median OS 39mo vs 27mo (ns) Statistically different at 2years but not at 6 years FU
WHO (Cascinelli et al. 2001)	III	IFN-a 3MU sc x3/wk x36mo	IFN=225 Obs=219	5yr DFS 28% vs 28% (p=0.5) 5yr OS 35% vs 37% (p=0.72)
UKCCR (AIMHIGH) (Hancock et al. 2004)	IIB-III	IFN-a 3MU sc x3/wk x24mo	IFN=338 Obs=336	DFS: HR 0.94 (0.75-1.18; p=0.6) OS: HR 0.91 (0.75-1.1; p=0.3)
EORTC18871 (Kleeberg et al. 2004)	IIA-III	IFN-a 1MU sc alt days x12mo	IFN=240 Obs=244	DFS: HR 1.04 (0.84-1.3; ns) OS: HR 0.96 (0.76-1.21; ns)

Trial	Stage	IFN regimen	No	Results
EORTC18952 (Eggermont et al. 2005)	IIB-III	1yr: IFN-a 10MU sc x5/wk x4wk then 10MU sc x3/wk x12mo 2yr: IFN-a 10MU sc x5/wk x4wk then 5MU sc x3/wk x24mo	IFN 1 yr=553 2 yr=556 Obs=279	DFS: 2yr vs obs: HR 0.83 (0.66-1.03; ns); 1yr vs Obs: HR 0.93 (0.75- 1.16; ns) OS: No differences between the 3 groups.
DeCOG (Garbe et al. 2008)	III	IFN-a 3MU sc x3/wk x24mo	IFN=148 Obs=148	4yr DFS 59% vs 42% (p=0.0045) 4yr OS 39% vs 27% (p=0.0018)
EORTC18991 (Eggermont et al. 2008)	III	Peg IFN-a 6ug/kg sc x1/wk x8wk then 3mg/kg sc x1/wk x60mo	IFN=627 Obs=629	DFS: HR 0.82 (0.71-0.96; p=0.01) OS: HR 0.98 (0.82-1.16; p=0.78)
Nordic (Mocellin et al. 2010)	IIB-III	1yr: IFN-a 10MU sc x5/wk x4wk then 10MU sc x3/wk x12mo 2yr: IFN-a 10MU sc x5/wk x4wk then 10MU sc x3/wk x24mo	IFN 1 yr=285 2 yr=286 Obs=284	DFS: 2yr vs obs: HR 0.83 (0.68-1.03; p=0.178); 1yr vs Obs: HR 0.77 (0.63-0.96; p=0.034) OS: No differences between the 3 groups.

Abbreviations: IFN-a: interferon alpha; PEG IFN-a: pegylated interferon alpha; DFS, disease free survival; RFS: relapse free survival; OS: overall survival; HR: hazard ratio; wk: week; mo: month; yr: year; sc: subcutaneous; iv: intravenous; im: intramuscular; Obs: observation.

Table 1. Adjuvant studies with Inteferon

An individual patient data (IPD) meta-analysis of 13 trials evaluated efficacy of IFN in high risk melanoma patients (Wheatley 2007). This showed a clear benefit of adjuvant IFN in terms of relapse-free survival (RFS) (HR 0.87, 0.81-0.93; p=0.00004) and an absolute benefit in overall survival of 2% at 10 years (HR 0.9, 0.84-0.97, p=0.008). There was no clear evidence of a difference in outcome by either dose or duration of IFN treatment.

A more recent review and meta-analysis has been published in 2010 and includes 14 trials in total (12 comparing IFN to observation) (Mocellin et al. 2010). This meta-analysis consists of the 12 studies contained in the 2003 report (4 with updated survival data) plus 2 more recent studies (DeCOG and E18991). This demonstrated a relapse-free survival advantage favouring IFN adjuvant treatment (HR 0.82, p<0.001). Original data from 12 of the 14 studies was used to assess overall survival. A statistically significant reduction in risk of death was seen for patients receiving IFN treatment (HR 0.89, p=0.002). No clear differences were identified according to IFN regimen or dose or stage.

Despite the small advantage in OS, the optimal dose and schedule has not been clearly defined. Many of the previous studies used different doses and schedules of IFN and they

varied in the inclusion criteria, particularly by stage of disease. The drug itself can have substantial toxicities especially at high doses and can reduce quality of life for the duration of treatment (Bottomley et al. 2009).

Subgroup analyses of adjuvant studies have attempted to define which group of patients who would be most likely to benefit. EORTC 18952 and EORTC 18991 trials stratified patients according to stage of disease and number of lymph nodes involved (Eggermont et al. 2008; Eggermont et al. 2005). Both these studies suggested that the greatest benefit of IFN is seen in stage II or III-N1 disease with reduced advantage of IFN with macroscopic nodal involvement (stage III-N2 disease)

A subgroup analysis of the meta-analysis published in 2007 (Ives et al. 2007) revealed the effect of IFN did not differ according to age, gender, disease site, stage, number of nodes or Breslow thickness. However, patients with ulcerated tumours appeared to have greatest benefit both in terms of disease-free and overall survival ($p < 0.03$). Combined post hoc analysis of EORTC 18952 and 18991 studies also demonstrated the greatest reductions in disease-free survival and overall survival in patients with ulcerated tumours (Eggermont 2009). This hypothesis will be tested further in the planned EORTC 18081 trial which will compare PEG-IFN α -2b to observation in patients with ulcerated primary melanoma and a Breslow thickness greater than 1mm.

The subsequent meta-analysis of the EORTC 18952 and 18991 studies showed that the benefit for interferon was confined to patients with ulcerated primary tumours. Conversely, unplanned subgroup analysis of the Nordic trial showed most benefit in patients with highest tumour burden before lymph node dissection and no relationship between benefit of IFN and ulceration of the tumour.

Pegylated interferon (PEG Intron) has been approved as an adjuvant therapy in 2010 on the basis of an improvement in DMFS in patients with resected sentinel node positive disease (Eggermont et al. 2008).

Autoimmunity, as defined by the development of serum autoantibodies and/or clinical vitiligo, has been investigated as a potential predictive factor of efficacy for IFN. In one study, patients receiving high dose IFN who developed autoimmunity (about a quarter of the patients) had an improved DFS and overall survival compared to those patients who did not (Gogas et al. 2006). However, other studies have failed to confirm any associations (Bouwhuis et al. 2009).

Vaccine strategies have also been evaluated in clinical trials, but to date the results have not been encouraging. Three studies (Cancervax stage III, Cancervax stage IV resected and EORTC 18961) have shown a survival disadvantage for these vaccines in this situation, and a further study (ECOG 1694) suggested a survival disadvantage for GMK vaccine. Studies with gp100 vaccines in advanced disease have shown no survival disadvantage (Schwartzentruber et al. 2009; Hodi et al. 2010). It is unclear why these adjuvant studies were negative but it encourages caution in employing long term vaccine based immune stimulation as an adjuvant therapy.

The adjuvant treatment of intermediate and high risk melanoma patients still varies greatly from country to country, with greater use of adjuvant interferon in USA and some North European Countries. Participating in a clinical trial remains the preferred option for these patients and most of randomised studies currently ongoing still include an observation arm. The DERMA trial (NCT00796445) is currently randomising MAGE-A3 positive patients with high risk (stage III) melanoma to receive a MAGE peptide vaccine plus adjuvant or placebo. A total of 1300 patients are planned to be enrolled. Prespecified subgroup analysis will

examine whether a previously identified genetic classifier can predict those likely to benefit from treatment (Kruit et al. 2008).

The European Organisation for the Research and Treatment of Cancer (EORTC) Melanoma Group is conducting a randomised double-blind phase III trial (EORTC 18071) evaluating Ipilimumab, a blocking antibody to CTLA4, versus placebo in patients with completely resected stage III disease (NCT00636168). Ipilimumab has recently been approved by the FDA for the treatment of advanced disease. A study comparing ipilimumab to high dose IFN in high risk disease is also planned (NCT01274338).

The AVAST-M study is a large randomised study enrolling across the United Kingdom. Patients with completely resected stage IIb-III melanoma are randomised to receive bevacizumab 7.5 mg/kg every 3 weeks for 1 year or observation. The planned sample is 1320 patients and the study aims to identify an 8% difference in overall survival. Relapse-free survival, quality of life, toxicity and biomarkers are secondary end points.

All 3 studies have nearly completed accrual and results will be available in the next 3-5 years, depending on the mix of risk groups included. The outcomes of these studies, and developments in treatment of advanced disease, will determine the landscape of adjuvant therapy and clinical trials over the next 5-10 years.

3. Advanced disease

The last 20 years have seen a huge body of work trying to improve outcomes for patients with advanced melanoma, with no real benefits. The median survival in recent chemotherapy studies has improved over historical controls, but in the main this has been due to better patient selection and stage migration due to improved imaging techniques. This is now changing very quickly.

Where once we could design clinical trials with an overall survival endpoint, confident that the outcome would not be affected by subsequent treatment, we now have a number of new drugs with either a proven survival benefit, or preliminary data to suggest that this will be the case. Here we review the pivotal clinical trials and their implications for treatment.

3.1 Standard chemotherapy

Single agent dacarbazine (DTIC) became the standard first-line chemotherapy based on a response rate of 10-20% in Phase II studies. No studies have ever been conducted to evaluate single agent chemotherapy against placebo or best supportive care. For this reason, the impact of treatment with DTIC on survival remains unclear.

Temozolamide (TMZ) is an oral alkylating agent and, together with DTIC, a prodrug of the active alkylating agent 5-(3-methyltriazene-1-yl) imidazole-4-carboximide (MTIC). Unlike DTIC, temozolamide spontaneously convert to MTIC.

A large phase III study compared overall survival of 305 chemo-naïve advanced melanoma patients treated with TMZ (200 mg/m²/d for 5 days every 28 days) or intravenous DTIC (250 mg/m²/d for 5 days every 21 days) (Middleton et al. 2000). The trial was designed to detect a 50% increase in survival compared with the 6 months expected with DTIC. Median progression-free survival (PFS) time was improved for temozolamide (1.9 v 1.5 months; HR 1.37; p=0.012). However, objective response was assessed every two cycles and this could explain the longer PFS interval for the temozolamide arm. Patients in the temozolamide arm experienced longer overall survival, which did not reach statistical significance (7.9 vs 5.7 months; p=0.054). There was no difference in response rates between the two arms (CR rate

of 2.6% vs 2.7% and PR rate of 10.9% vs 9.4% for temozolamide and DTIC arm, respectively). Both treatments were well tolerated. Most common grade 3/4 haematological toxicity was thrombocytopenia. Quality of life (QoL) at 12 weeks (QLQ-030 scores) showed a statistically significant difference favouring TMZ for physical functioning, fatigue, and insomnia. This trial demonstrated that TMZ represents an effective alternative to DTIC with potential advantage in QoL. Temozolamide did not receive regulatory approval for use in advanced melanoma, but there is a large use for off-label temozolamide chemotherapy, particularly in patients with brain metastases.

A large study conducted by the European Organization for Research and Treatment of Cancer (EORTC 18032) (Patel et al. 2008), randomized 859 patients with untreated stage IV melanoma, performance status 0-1 and LDH \leq 2x ULN to receive TMZ 150 mg/m² day 1-7 repeated every 14 days ("week on-week off") or DTIC 1000 mg/m² every 3 weeks. Both treatments were given until progression. The extended schedule allowed a 2.1 fold higher cumulative dose than the standard 5-days regimen and was thought to prolong depletion of the DNA repair enzyme MGMT, a known mediator of chemoresistance to temozolamide. Overall survival, did not differ between TMZ and DTIC (9.13 vs 9.36 months, $p=1.0$). There was also no difference in PFS (2.3 vs 2.17 months, $p=0.27$) and response rate (14% vs 10%, $p=0.05$) between the two groups. Thrombocytopenia was higher in the TMZ arm but there were no differences in non-haematological toxicities.

Despite well tolerated, the extended schedule temozolamide regimen confers no survival advantage over standard DTIC.

3.2 Biochemotherapy vs chemotherapy

Several randomized trials have evaluated chemotherapy combined with immunotherapy (IFN \pm IL-2; ie, biochemotherapy) in an attempt to improve both response rates and overall survival (OS) in the advanced setting. A metaanalysis by Ives et al, evaluated the effect of adding interferon- α (IFN α) \pm interleukin-2 (IL-2) to chemotherapy in patients with metastatic melanoma (Ives et al. 2007). Data was extracted from 18 trials with more than 2600 patients. Eleven trials (1395 patients) evaluated chemotherapy \pm IFN and seven trials (1226 patients) evaluated chemotherapy \pm IFN and IL-2.

Patients treated with biochemotherapy had increased PR (odds ratio = 0.66; 95% CI, 0.53 to 0.82; $p=0.0001$), CR (odds ratio = 0.50; 95% CI, 0.35 to 0.73; $p=0.0003$), and overall response rate (odds ratio = 0.59; 95% CI, 0.49 to 0.72; $p<0.00001$). There was a significant increase in overall response rate (ORR) for both the immunotherapy subgroups; IFN (odds ratio = 0.60; 95% CI, 0.46 to 0.79; $p=0.0002$) and IFN+IL-2 (odds ratio = 0.58; 95% CI, 0.44 to 0.77; $p=0.0001$). For PR (test for heterogeneity between subgroups; $p=0.08$) and CR ($p=0.007$) there was some evidence of a difference in treatment effect dependent on the type of immunotherapy used (IFN or IFN + IL-2). Biochemotherapy delayed the time to disease progression (odds ratio = 0.80; 95% CI, 0.71 to 0.89; $p=0.0001$), with no evidence of heterogeneity between individual trials or between the type of immunotherapy used. Despite improvement in response rate and PFS, there was no benefit for biochemotherapy on OS (odds ratio = 0.99; 95% CI, 0.91 to 1.08; $p=0.9$). Toxicity data was available only from 11 trials. Hematological toxicity was higher in the biochemotherapy arm but the treatment-related deaths rate was similar (0.6% vs 0.9%, $p=0.6$) for biochemotherapy group and chemotherapy group, respectively. These data suggest no benefit from adding IL-2 or IFN to chemotherapy.

3.3 Single agent vs combination chemotherapy

A number of combination chemotherapies have been evaluated for advanced melanoma. These regimens seemed promising in II trials, but failed to show survival advantage over DTIC alone when tested in randomized phase III trials.

The two most active combinations are the four-drug combination of cisplatin, DTIC, carmustine (BCNU), and tamoxifen (the Dartmouth regimen) and cisplatin, vinblastine, and DTIC (the CVD regimen).

The Dartmouth regimen was initially evaluated in 42 patients and demonstrated a response rate of 54% and overall survival of 412 days (Lattanzi et al. 1995). The Dartmouth regimen was then evaluated against DTIC in a large (n=240) phase III randomized trial (Chapman et al. 1999). Patients receiving the Dartmouth regimens achieved higher response rates, but the difference was not statistically significant (18.5% vs 10.2%, p=0.09). Median survival time from randomization was 7 months and there was no difference in survival time between the two treatment arms. Bone marrow suppression, nausea/vomiting, and fatigue were significantly more common in the Dartmouth arm. This trial demonstrated that polichemotherapy did not improve survival compared to single agent chemotherapy and crowned DTIC as the standard regimen in advanced Melanoma.

The CVD regimen was evaluated in a phase II trial in 52 patients (Legha et al. 1989). Overall response rate was 40% and median survival was 9 months. The treatment was associated with significant nausea, vomiting and diarrhea. On the basis of these promising results, a phase III trial evaluating CVD vs DTIC was conducted and showed a response rate of 24% and 11%, respectively with no difference in median survival between the two groups (6.7 vs 5.2 months) (Buzaid et al. 1993). (Results of the trials evaluating combination chemotherapy are summarized in table 2)

Because of the lack of improvement in overall survival and the worse toxicity achieved with polichemotherapy regimens, DTIC remains the preferred option as first-line chemotherapy in advanced/metastatic melanoma patients.

Author	Regimen	No	Response rate (%)	OS (mos)
Lattanzi (Lattanzi et al. 1995)	DTIC+C+BCNU	16	25	412 days
	DTIC+C+BCNU+ Tam (Dartmouth)	26	54	412 days
Chapman (Chapman et al. 1999)	Dartmouth	119	18.5	7.7
	DTIC	121	10.2	6.3
Legha (Legha et al. 1989)	CVD	52	40	12
Buzaid (Buzaid et al. 1993)	CVD	46	24	6.7
	DTIC	45	11	5.2

Abbreviations: DTIC: Dacarbazine; C: Cisplatin; BCNU: carmustine; Tam: Tamoxifen; CVD: cisplatin, vinblastine, dacarbazine; Dartmouth: dacarbazine + cisplatin + carmustine + tamoxifen; OS: overall survival; mos: months

Table 2. Phase II-III trials of polichemotherapy for advanced melanoma.

4. Immunotherapy

Melanoma cells can express a number of antigens that excite an immune response, as borne out by the better prognosis seen in patients with evidence of immune activation (vitiligo), the occasional case of spontaneous regression, the better outcome for patients with unknown primary (Chang and Knapper 1982; Lee et al. 2008), the increased incidence of melanoma seen in immunocompromised patients, and the response to immunotherapy agents including interferon alpha and interleukin 2.

4.1 Adoptive cell therapy

The ability to isolate and characterize anti-tumor lymphocyte has enabled the identification and characterization of multiple melanoma-associated antigens that can represent target of immunotherapy. Adoptive cell therapy (ACT) describes an immunotherapy approach in which tumor infiltrating lymphocytes (TIL) are harvested from fresh tumour tissue, expanded *ex vivo* and then reinfused after the patient has been lymphodepleted. Critical components of this complex process include the number, age and type of TILs, the degree of immunosuppression of the host, and the use of IL-2 after reinfusion.

Dudley et al, reported on 3 consecutive trials involving 93 patients in total (Dudley et al. 2008). In the first study, 43 patients received non-myeloablative chemotherapy consisting of cyclophosphamide and fludarabine before the TIL transfer. On the two next trials, patients were treated with 2 days of cyclophosphamide (60 mg/kg) plus 5 days of fludarabine (25 mg/m²) followed by a single fraction of 2 Gy of total body irradiation (TBI) (n=25) or 12 Gy of TBI (n=25). On the day following the final dose of TBI, patients received cell infusion with TIL and started high-dose IL-2 consisting of 720,000 IU/kg intravenously every 8 hours to tolerance. One or 2 days after TIL infusion, cryopreserved CD34+ hematopoietic stem cells were infused intravenously. Thirty seven percent of the patients had received previous chemotherapy and 83% had received previous IL-2. The response rates in these 3 studies were 48.8 %, 52% and 72%, respectively. Response of the marker lesions was seen in all visceral and soft tissue sites, including brain. Ten patients achieved a complete response with no relapses at 31-month follow-up. The median follow-up of these trials was 45, 27 and 10 months, respectively and there was no difference in survival between the groups (p=0.13) bearing in mind that the follow-up of the third trial is quite short. These studies demonstrate that ACT has the potential of improve the outcomes of a highly selected patient population, but its use at present should remains strictly as part of clinical trials.

4.2 Cytotoxic T lymphocyte-associated antigen 4 (CTLA 4) blockade

CTLA 4 is a key molecule in T-cell tolerance that serves as a natural braking mechanism for T-cell activation, allowing a return to homeostasis following an immune response. T-cell activation requires engagement of the T-cell receptor to antigen-bound major histocompatibility complex (MHC) on the antigen-presenting cell (APC) as well as engagement of the costimulatory molecule CD28 on the T-cell surface by members of the B7 family on the APC. Following T-cell activation, CTLA 4 cell-surface receptors are upregulated and compete with CD28 for binding to B7 sending an inhibitory signal that downregulates T-cell activation.

This led to the hypothesis that blocking the CTLA 4-B7 interaction would lead to enhanced and prolonged T-cell activation with subsequent more vigorous antitumor immune response.

Two anti-CTLA 4 blocking antibodies have been evaluated in clinical trials: tremelimumab (formerly CP-675,206 or ticilimumab) and ipilimumab (formerly MDX010)

4.3 Tremelimumab

Tremelimumab is a fully human IgG2 monoclonal antibody specific for human CTLA 4. A Phase II study in 251 previously treated patients with advanced disease reported a median survival of 10 months (Kirkwood et al. 2010)

A phase III trial randomized 655 patients with unresectable stage IIIC and stage IV melanoma without brain metastasis and LDH below twice the upper limit of normal to receive tremelimumab 15 mg/kg every 3 months vs chemotherapy (TMZ 200 mg/m² p.o. d1-5 q28d or DTIC 1,000 mg/m² IV q21d) (Ribas et al. 2008). The study was stopped early by the data safety monitoring committee after the second interim analysis because of futility. The median OS by was 11.8 months (95% CI 10.4, 13.9) in the tremelimumab arm, and 10.7 months (95% CI 9.3, 12.0) in the chemotherapy arm. Subgroup analysis of this trial has suggested that patients with low baseline C-reactive protein had improved survival (median OS: 19.1 vs 12.7 months, $p = 0.0037$) with tremelimumab compared with chemotherapy. (Marshall, Ribas, and Huang 2010)

4.4 Ipilimumab

Ipilimumab is a fully human monoclonal immunoglobulin (IgG1k) specific for CTLA-4 that was investigated in three phase II trials (study 007, 008, and 022), enrolling a total of 487 treatment-naïve and pretreated patients with unresectable stage III or stage IV melanoma. Study 007 randomized 115 pretreated and treatment naïve patients to receive ipilimumab (10 mg/kg every 3 weeks, for four doses) with prophylactic budesonide/placebo to prevent colitis (Weber et al. 2009) Budesonide did not affect the rate of grade ≥ 2 diarrhea and median survival was 17.7 vs 19.3 months for the budesonide and placebo group, respectively. The one-year survival was greater than 55%.

Study 008 was a single-arm phase II trial investigating ipilimumab at the dose of 10 mg/kg in 155 pretreated patients (O'Day et al. 2010). Best overall response rate (BORR) was 5.8% with a disease control rate (DCR) of 27% and a median overall survival of 10.2 months. The 1-year survival rate was 47.2% and ongoing survival analyses showed 18- and 24-month survival rates of 39.4% and 32.8%, respectively. Most common adverse events (AEs) were immune-related, occurring mainly in the skin (grade 3/4: 3.2%) and gastrointestinal tract (grade 3/4: 8.4%). Study 022 was a randomized, double blind, phase 2 trial that assessed three different doses of ipilimumab (0.3 mg/kg, 3.0 mg/kg, or 10 mg/kg) in 217 pretreated patients (Wolchok et al. 2010). Patients were treated with four 3-weekly doses (induction phase), then patients without PD at week 24 were eligible to continue their assigned dose of ipilimumab every 12 weeks (maintenance phase). Only 20 patients received maintenance therapy. Patients progressing after an initial response or stable disease were allowed to undergo reinduction with ipilimumab at the dose of 10 mg/kg. There was a clear dose response with a statistically significant improvement in best overall response rate with the 10 mg/kg dose (BORR 11.1%). Despite the study was not designed to detect differences in survival, there was a trend for improved overall survival favoring the dose of 10 mg/kg that did not reach statistical significance (8.6 vs 8.7 vs 11.4 months). Most common AEs (skin rash and diarrhea) were immune-related and the frequency rose with increasing dose of ipilimumab. The authors concluded that the dose of 10 mg/kg warranted further evaluation.

Phase II trials of ipilimumab in advanced melanoma are summarized in table 3.

Study	Dose (mg/kg)	Population	No	ORR (%)	DCR (%)	MS (mos)	1y-S (%)
007	10 + budesonide	pre-treated and untreated	58	12	31	17.7	55.9
	10 + placebo		57	16	35	19.3	62.4
008	10	pre-treated	155	5.8	27	10.2	47
022	0.3	pre-treated	73	0	13.7	8.6	39.6
	3	pre-treated	72	4.2	26.4	8.7	39.3
	10	pre-treated	72	11.1	29.2	11.4	48.6

Abbreviations: No: number; ORR: overall response rate; DCR: disease control rate; MS: median survival; mos: months; 1y-s: 1-year survival

Table 3. Phase II trials of ipilimumab in advanced Melanoma

A recently published large phase III trial randomized 676 pretreated HLA-A*0201-positive patients with unresectable stage III or IV melanoma to receive, in a 3:1:1 ratio, ipilimumab plus gp100 (n=403 patients), ipilimumab alone (n=137), or gp100 alone (n=136). (Hodi et al. 2010). Ipilimumab was administered at the dose of 3 mg/kg every three weeks, for four cycles. Patients with stable disease for 3 months after week 12 or a confirmed partial or complete response were offered additional courses of therapy (reinduction) with their assigned treatment regimen, at disease progression. The original primary endpoint was the best overall response rate but this was amended to overall survival. Seventy seven (out of 82) patients had central nervous system metastasis at baseline and received at least one dose of ipilimumab. The median overall survival was 10.0 vs 10.1 vs 6.4 months for the ipilimumab-gp100, ipilimumab alone and gp100 alone arms, respectively.

There was no difference in overall survival between the two ipilimumab groups (HR 1.04; p=0.76). The median progression-free survival was 2.76 months in the ipilimumab-gp100 group, 2.86 months in the ipilimumab-alone group, and 2.76 months in the gp100-alone group. One-year survival and 2-year survival for ipilimumab-plus-gp100 group, the ipilimumab-alone group, and the gp100-alone group were 43.6%, 45.6%, 25.3% and 21.6%, 23.5%, 13.7%, respectively. Consistently with phase 2 data, most common adverse events were immune-related and most affected were skin and gastrointestinal tract. Other serious AEs included hepatitis and endocrinopathies. Grade 3/4 colitis occurred in 3-5% of the patients treated in the ipilimumab-gp100 and ipilimumab-alone arm, respectively. There were 14 treatment-related deaths (2.1%), of which 7 were associated with immune-related adverse events. This study was restricted to patients that were HLA-A2 positive, because the vaccine is presented in a HLA restricted fashion. However, CTLA-4 blockade by ipilimumab is independent of HLA status therefore HLA-typing patients who are suitable to receive ipilimumab is not necessary.

This trial is, so far, the only trial showing a survival advantage in the history of melanoma and has set a new standard of care. On the basis of these results, US Food and Drug Administration (FDA) has recently approved ipilimumab for treatment of unresectable or metastatic melanoma.

Bristol Myers Squibb has also announced that a first-line trial (CA180-024) has met its primary end point demonstrating improvement in overall survival with ipilimumab in combination with dacarbazine vs dacarbazine alone. Data will be presented at the forthcoming ASCO Annual Meeting 2011 and are eagerly awaited.

4.5 Immune-related response criteria

The pattern and duration of responses associated with CTLA 4 blockade differ from those associated with cytotoxic agents. Objective responses to ipilimumab may not occur until the post-induction period of therapy and may occur, in some cases, after PD as defined by WHO or RECIST criteria.

Four patterns of response to ipilimumab have been identified: 1) initial response in baseline lesions, 2) stable disease with subsequent slow and steady decline in total tumor volume, 3) response after increase in total tumor volume, 4) reduction in total tumor burden after the appearance of new lesions.

The apparent increase in tumor burden that sometimes happens before an objective response could be either related to continue tumor growth until a sufficient immune response develops or transient immune cells infiltrate that can cause edema.

In order to provide a more comprehensive assessment of clinical activity of these agents, the immune-related response criteria (IrRC) have been developed as a variation of WHO criteria (Wolchok et al. 2009). Using these criteria, new lesions do not always represent progressive disease, and the criteria for calculating response or progression have been modified. The immune-related response criteria may help to explain why patients with apparent PD by the traditional response criteria go on to experience long-term survival.

5. Targeted agents

The identification of an activating mutation in braf in 50-60% of cutaneous melanoma samples (Davies et al. 2002), and the fact that in this was primarily a single codon mutation, resulting in the V600E mutation in the vast majority of cases, opened up the field to the potential for targeted therapy. Since then, it has been shown that approximately 50-70% of cutaneous melanomas have a mutation in the MAP kinase pathway, either BRAF or NRAS, and approximately 20% of acral lentiginous melanomas have a mutation in c-kit (Curtin et al. 2005; Curtin et al. 2006). The identification of potential targets resulted in the design of a number of targeted therapies, which have been evaluated in clinical trials.

5.1 Sorafenib

Sorafenib (Nexavar[®], Bayer Healthcare Pharmaceuticals) is a multikinase inhibitor with potent non-selective action against RAF1, with additional broad-spectrum activity against VEGFR-2, VEGFR-3, PDGFR- β , the tyrosine kinase FLT3 and KIT receptors. It is one of the first small molecules to be tested in the treatment of melanoma.

Its use as a single agent treatment did not seem to confer any meaningful benefit in melanoma patients (Eisen et al. 2006). A phase II trial investigating the combination of sorafenib/placebo and dacarbazine showed promising results, with an improvement in progression-free survival (21.1 weeks vs 11.7 weeks, HR 0.665; p=0.068 for sorafenib and placebo arm, respectively) but no improvement in overall survival (45.6 vs 51.3 weeks HR=1.022; p=0.927, for sorafenib and placebo, respectively) for patients receiving dacarbazine/sorafenib combination (McDermott et al. 2008).

Two large phase III trials investigated sorafenib in combination with carboplatin/paclitaxel chemotherapy.

The PRISM study evaluated carboplatin/paclitaxel (CP) with sorafenib/placebo in a phase III randomized study as a second-line treatment for unresectable stage III or stage IV melanoma patients. There was no improvement in either median PFS (17.9 vs 17.4 weeks, HR=0.91; p=0.49, for CP/placebo and CP/sorafenib, respectively) or median OS (42.0 weeks for both arms, p=0.92) (Hauschild et al. 2009). More recently, results from a first-line phase III trial (E2603 intergroup trial) have been reported. This trial randomized 823 previously untreated patients with metastatic melanoma, to receive sorafenib or placebo in combination with CP. The study crossed the futility boundaries and therefore was stopped and unblinded after 75% of the events required for the final analysis were reached. There was no improvement in median OS (11.0 vs 11.3 months in sorafenib and placebo, respectively, p=N/A), and no difference in median PFS (4.9 vs 4.1 months, p=0.48 for sorafenib and placebo, respectively (Flaherty, Lee et al. 2010).

5.2 BRAF selective inhibitors

Vemurafenib (RG7204, PLX4032), is an orally available inhibitor of mutated BRAF has the most clinical evidence accrued thus far. Preliminary data from the phase I trial of PLX4032 was reported in 2010 in the *New England Journal of Medicine* (Flaherty, Puzanov et al. 2010). The trial enrolled 55 patients, of which 49 diagnosed with melanoma, in a dose escalation phase and further 32 patients harbouring the BRAF V600E mutation enrolled in an extension phase receiving the recommended dose of 960 mg twice daily. Among the 16 patients with melanoma receiving 240 mg twice a day or more the overall response rate was 69%. Five patients with metastatic melanoma without BRAF mutations were treated with no evidence of tumour response. Notably, in the extension phase, 26 out of 32 (81%) patients treated had an objective response. The estimated median progression-free survival in these patients is 7 months and the estimated median overall survival has not yet been reached. The most common grade 2 or 3 adverse events were arthralgia, fatigue and skin-related toxicity including 18 patients with cutaneous squamous cell carcinomas (keratoacanthoma type) treated with excision without interruption of treatment. Of the adverse events observed, 89% were grade 1 and 2.

The exciting results showed in the phase I trial prompted the design of the phase II trial of RG7204 in previously treated patients with metastatic melanoma harbouring a BRAF V600E mutation (BRIM 2 trial). This data was presented by Sosman and colleagues at The Seventh International Melanoma Research Congress of the Society for Melanoma Research, in November 2010 (Sosman et al. 2010). BRIM 2 (NCT00949702) was a single arm, multicentre, open label phase II trial in which 132 patients received RG7204 until progression. Primary endpoint was overall response rate. The presence of the V600E BRAF mutation was confirmed with the cobas® 4800 BRAF V600 mutation test developed by Roche. The ORR was 52% and 30% of patients achieved stable disease. The median PFS was 6.2 months and, as with the phase I trial, median overall survival has not yet been reached. Consistently to what observed in the phase I trial, most common adverse events observed were arthralgia and skin toxicity, including 26% grade 3 cutaneous squamous cell carcinoma. However, fourteen percent of the patients also experienced grade 3 abnormalities in liver function tests and 10% Grade 3 dysphagia and pancreatitis

The confirmation of clinical efficacy from the phase two trial led to the design of BRIM 3 (NCT01006980), a large phase III randomized trial evaluating RG7204 head to head with

dacarbazine in previously untreated patients with metastatic melanoma harbouring BRAF V600E mutation. This study has recently terminated accrual and a recent press release from Roche has showed that RG7204 met its co-primary endpoints. Patients treated with the BRAF selective inhibitor have achieved longer OS and PFS than the chemotherapy arm. Final data will be presented at the ASCO Annual Meeting 2011 (Roche 2011). Since the interim analysis data, the trial protocol has been amended so that patients in the dacarbazine arm who have progressed have now the option to crossover to receive RG7204. Other BRAF selective inhibitors are now in clinical development. GSK2118436 is a potent oral selective ATP competitive BRAF inhibitor. The results from phase I/II dose escalation study of GSK2118436 in melanoma and other solid tumours were reported at ASCO 2010. When reported, maximum tolerated dose had not been reached but clinical activity with minimal toxicity was seen. The most common grade 3/4 toxicities reported were skin changes, headache, nausea, fatigue and vomiting and low grade squamous cell carcinoma in order of reducing frequency. Notably, 79% of patients with BRAF mutation were shown to have exposure-related decrease in FDG-PET metabolic uptake and 60% had a >20% reduction in tumour size by RECIST at initial restaging (Kefford et al. 2010).

A phase III study is ongoing and will compare GSK2118436 to dacarbazine (DTIC) in previously untreated subjects with BRAF V600E mutation positive advanced (Stage III) or metastatic (Stage IV) melanoma. (NCT01227889). Another phase II trial is evaluating GSK2118436 in BRAF Mutant stage IV melanoma with brain metastases (NCT01266967). The RAS/RAF/MEK/ERK pathway is shown in figure 1.

5.3 MEK inhibitors

MEK 1 and 2 belong to a family of dual specificity kinases which are downstream of RAF in the mitogen activated protein kinase signalling cascade (Crews, Alessandrini, and Erikson 1992; Rosen et al. 1994). ERK1/2 is constitutively active in melanoma cells regardless of their BRAF or NRAS status, presenting a potential target for treatment in this population. Phosphorylated ERK is important for melanoma because it plays key roles in cell cycle entry, invasion, and possibly angiogenesis, as well as in resistance to apoptosis (Smalley 2003). AZD6244 is a highly selective allosteric inhibitor of MEK1/2. Treatment with AZD6244 leads to suppression of pERK levels in melanoma in a manner independent of BRAF and NRAS mutation status (Haass et al. 2008). Single agent therapy of melanoma with AZD6244 has been disappointing.

The interim results from a phase 2 study of the safety and efficacy of AZD6244 versus temozolomide in patients with advanced melanoma were reported in 2008. This was an open label randomised multicentre phase 2 study which randomized 200 patients with untreated stage III or IV melanoma to receive AZD6244 or temozolamide. There was no difference in PFS between the two treatment arms in the overall population (HR 1.07; 80% CI 0.86-1.34) or in the BRAF mutant subgroup (HR 0.85; 80% CI 0.58-1.24). Overall survival data are immature (67 deaths) but the interim analysis showed no difference between the two arms in the overall population (HR 1.23; 80% CI 0.88-1.71). Mutation status was assessed in archival tissue and 67 (50%) cutaneous melanoma patients had a BRAF mutation and 24 (18%) patients had a mutation in NRAS. In BRAF mutant patients, the HR estimate for OS favoured AZD6244 (HR 0.68; 80% CI 0.38-1.21) (Dummer et al. 2008).

A randomised phase II study (AZD Study 6, NCRN063) that randomised 91 BRAF V600E mutant patients to receive DTIC + AZD6244/placebo has completed accrual in 2010 and the results are awaited.

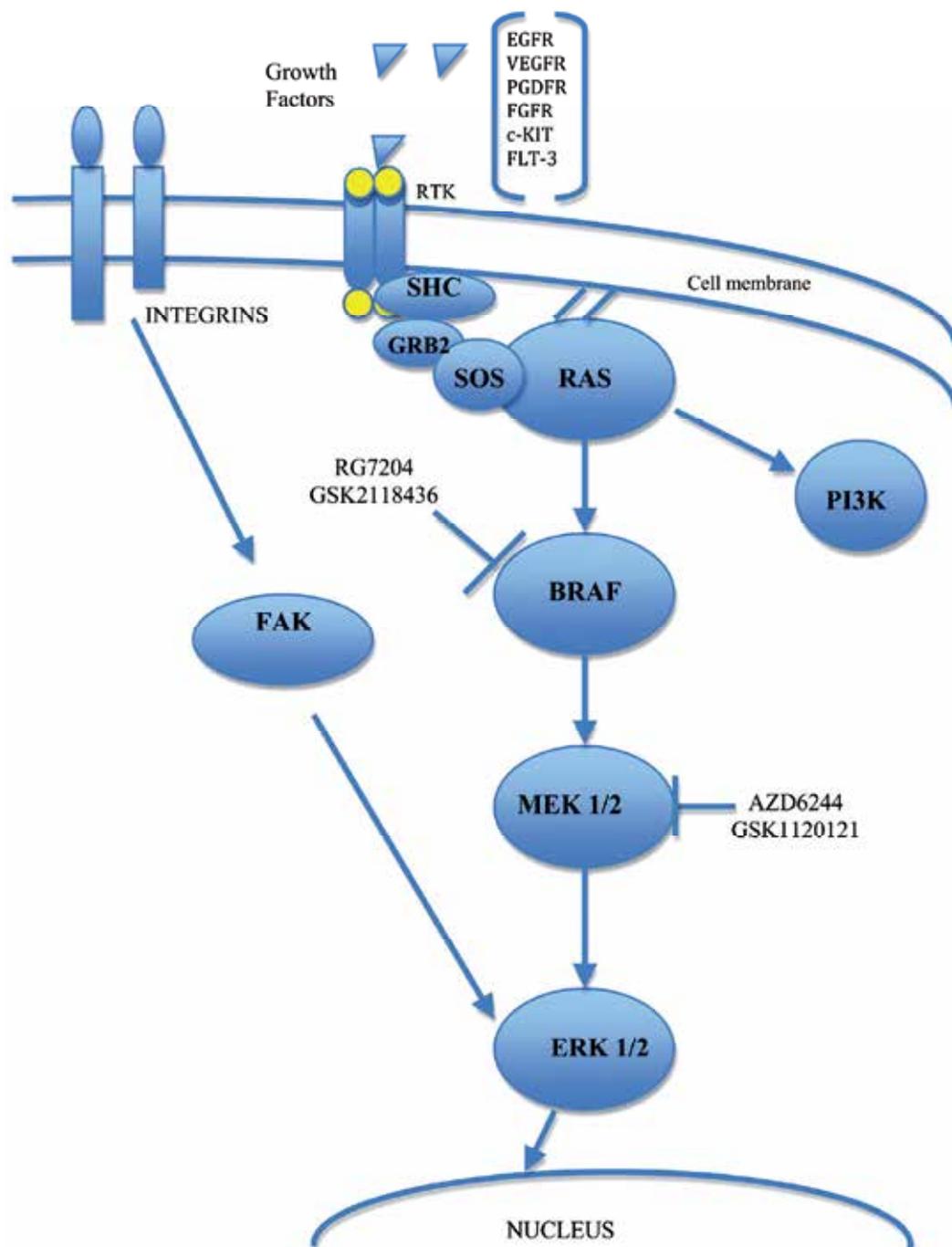


Fig. 1. The RAS/RAF/MEK/ERK pathway

AZD6244 and docetaxel have demonstrated synergy in a variety of animal xenograft models, including melanoma. Furthermore, a patient with wild type BRAF and NRAS treated with AZD6244 showed a response to treatment in the initial study. A phase 1 study of the combination of AZD6244 and docetaxel has been conducted (Astrazeneca study D1532C00004) in 12 patients with melanoma: 1 had a complete response; 1 a partial response; 4 had stable disease beyond 6 cycles of treatment (18+ weeks) indicating clinical activity worthy of further study.

DOC-MEK is a first-line randomised, double-blind placebo controlled phase 2 trial, that randomizes BRAF wild-type stage IV melanoma to receive docetaxel 75mg/m² IV and placebo/AZD6244.

GSK1120212 is a potent and selective allosteric inhibitor of the MEK1/2 enzymes. A three-part phase I trial evaluating GSK1120212 in 84 patients with advanced solid tumors and lymphoma was reported at ASCO 2010 (Infante et al. 2010). This trial included 29 melanoma and 15 pancreatic cancer patients. The maximum tolerated dose was found to be 3 mg OD and recommended phase II dose (RP2D) chosen was 2 mg OD. At doses \geq RP2D (n=77), the most common adverse events were rash (30% G2, 5% G3) and diarrhea (9% G2, 3% G3). In the 20 evaluable melanoma patients with known BRAF status, 5 PR were observed, all having \geq 50% tumor reduction; 3 have stayed \geq 30 weeks on study, the other 2 are ongoing. In the 11 BRAF mutant melanoma patients, 3 PR, 5 SD (including a patient previously treated with the BRAF inhibitor RG7204), and 3 PD were observed. Two of the 3 PD were due to new brain lesions. In 9 BRAF wild-type patients with melanoma, 2 PR, including a patient with a GNAQ mutation, and 3 SD were achieved.

A Phase II open-label, multi-site study to investigate the objective response rate, safety, and pharmacokinetics of GSK1120212 in subjects with BRAF mutation-positive melanoma who were previously treated with or without a BRAF inhibitor has completed accrual and is ongoing.

The recently initiated METRIC Phase 3 study will compare (2:1 randomisation) GSK1120212 vs DTIC (untreated patients) or paclitaxel (previously treated patients) in advanced or metastatic melanoma harbouring BRAF V600 E/K mutation. The primary endpoint is PFS and subjects who have progression on chemotherapy will be offered the option to cross over to receive GSK1120212.

5.4 Bevacizumab

Bevacizumab is a monoclonal antibody that acts indirectly to prevent angiogenesis by binding the VEGF-A receptors, thus preventing the interaction with VEGFR2. Melanoma cells, when exposed to chemotherapy agents, overproduce VEGF thus encouraging the development of chemoresistant tumour phenotypes. There is evidence of increased production of angiogenic factors with more advanced stage of melanoma (Lev et al. 2004; Ugurel et al. 2001). For this reason bevacizumab was investigated as a treatment for melanoma.

A number of phase II trials with bevacizumab in combination with chemotherapy have been reported so far. The BEAM trial randomized (2:1) 214 patients to carboplatin/taxol + bevacizumab or placebo, in the first-line setting. There was an improvement in progression-free survival (primary endpoint) in the bevacizumab arm compared to placebo (5.6 months vs 4.2 months, HR 0.78, p=0.16), which did not reach statistical significance. Notably there was a non statistically significant improvement in OS (secondary endpoint) (12.3 vs 9.2 months, HR=0.79, P=0.19) (O'Day, Sosman, and Peterson 2009)

A phase II trial evaluated the use of bi-weekly bevacizumab in combination with carboplatin and weekly paclitaxel in 53 previously treated patients. The median PFS and OS were 6 and

12 months, respectively. There was a high incidence of haematological toxicities, most common being grade ≥ 3 neutropenia (53%), leukopenia (38%) and thrombocytopenia (11%). There were also 40 episodes of bleeding in 31 patients, including two grade 2 bronchopulmonary haemorrhages and one grade 5 central nervous system hemorrhage (Perez et al. 2009).

A Phase II study of temozolomide 150 mg/m² for 7 days and bevacizumab 10 mg/kg every 2 weeks in 62 patients reported a median PFS of 4.2 months and OS of 9.3 months (Dummer et al. 2010).

5.5 Oblimersen

Oblimersen sodium (Genasense; Genta International Inc, Berkeley Heights, NJ) is a 18-base phosphorothioate antisense oligonucleotide that binds to the first 6 codons of the Bcl-2 mRNA open reading frame (Klasa et al. 2002). Overexpression of Bcl-2 may be partially responsible for the multi drug resistance seen in melanoma (Soengas and Lowe 2003). In a randomised controlled trial reported by Bedikian et al, 771 chemo-naive advanced melanoma patients were randomised to receive either dacarbazine (1000 mg/m² on day 1) and oblimersen (7 mg/kg/day as a continuous infusion for 5 day) (n=386) or dacarbazine alone (n=385) (Bedikian et al. 2006). There was a trend for longer OS in the oblimersen arm which did not reach the statistical significance (9 vs 7.8 months, HR= 0.87, 95% CI=0.75-1.01, p=0.077). A subgroup analysis for patients with normal LDH showed significantly improved overall survival, PFS and overall response rate in favour of the oblimersen-dacarbazine group. On the basis of these results, a phase III trial (AGENDA trial) has evaluated dacarbazine with or without Genasense in Advanced Melanoma with low LDH level. The initial results showed no impact of the addition of oblimersen to chemotherapy on OS or PFS, but a more mature analysis with longer follow-up is awaited (Genta 2009).

After many years of little or no improvement in outcomes for patients with melanoma, the tides are changing with 2 new agents, either licensed or about to be, showing significant activity in advanced disease. The results of 3 large randomised adjuvant studies will also report in the next few years, hopefully also improving the treatment options for high risk patients. The management algorithm is being rewritten, but is generating more questions than answers. We have little understanding of resistance mechanisms, optimum scheduling, use in combination, long term toxicities etc – these will be the questions addressed in trials over the next 5 years.

Five years ago, we could only have dreamed of being in the position to have to consider these issues. The paradigm of basic scientific research discoveries being explored further in translational studies, then validated in rigorous clinical trials is exemplified by the progress achieved melanoma treatment.

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Part 7

Treatment of Oral and Uveal Melanoma

Oral Malignant Melanoma

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1. Introduction

It is generally accepted that the outcome of oral malignant melanoma is worse than that of cutaneous and systemic head and neck melanoma. The five-year survival reported in the literature for oral melanomas varies from 0 - 20 % (Liversedge, 1975, Rapidis et al., 2003, Rapini et al., 1985), whereas the overall survival for head and neck melanomas ranges between 20 and 48 % (Guzzo et al., 1993, Patel et al., 2002, Stern and Guillaumondegui, 1991, Temam et al., 2005), confirming anatomical site specific variation.

Treatment modalities for primary oral melanoma include surgical resection with or without neck dissection. Adjunctive modalities such as immunotherapy, chemotherapy and radiation therapy may offer a supportive but as yet non curative role. The mainstay of curative treatment is surgery, mandating complete resection of the tumor with clear margins if possible (Rapini et al., 1985, Umeda and Shimada, 1994). Immunotherapy and chemotherapy are techniques which are the subject of controlled trials or palliation (Mendenhall et al., 2005). The rarity of oral malignant melanomas means that to date no randomized clinical trials have been conducted to establish an evidence base in the literature to provide comparisons of treatment modalities. The efficacy of surgery with planned supplementary radiotherapy remain unresolved and continue to generate controversy (Patel et al., 2002).

Due the rarity of this disease, there is a lack of consistency in immunohistochemical confirmation of the diagnosis, staging strategies and treatment planning as diagnostic techniques and treatment have evolved over recent years in melanoma in other body sites and attempts have been made to translate these to the much rarer oral melanoma. This book section would like to aid the reader in understanding the current state of etiology, epidemiology, and treatment modalities of this very rare but aggressive tumor entity.

2. Oral malignant melanoma

The diagnosis of oral malignant melanoma often remains difficult. Differential diagnoses should include benign as well as malignant lesions and exogenous pigmentations can be often found (Figure 1). It also needs to be borne in mind that amelanotic malignant melanoma can affect the mouth, comprising one third of all oral malignant melanomas.



Fig. 1. Oral pigmentation due to inoculation of amalgam after dental treatment in the left buccal mucosa.

The histological spectrum of benign pigmentations is wide: macular hyperpigmentation caused by junctional proliferation with or without cellular atypica, melanocytic naevi (Figure 2), such as junctional, compound, subepithelial, blue and combined naevi. Other causes of oral pigmentation include: race, Peutz-Jeghers syndrome (Daley and Armstrong, 2007), Laugier-Hunziker syndrome (Mowad et al., 1997), Addison's disease (Lamey et al., 1985), patients with pulmonary diseases, especially in lung cancer (Merchant et al., 1976), and hemosiderosis.



Fig. 2. Melanocytic naevi in the upper left vestibule.

Some authors describe a preexisting oral pigmentation for several month or years in one third of all patients with a primary oral malignant melanoma (Chaudhry et al., 1958, Liversedge, 1975, Rapini et al., 1985), although others found no correlation (Meleti et al., 2007). The appearances of oral malignant melanomas vary and should always be considered in oral lesions without the tendency to heal (Figure 3). Remember that about 1/3 of all oral malignant melanomas are not pigmented (Anneroth et al., 1973, Chaudhry et al., 1958, Greene et al., 1953, Lengyel et al., 2003, Liversedge, 1975, Rapidis et al., 2003).



Fig. 3. Intraoral view of a malignant melanoma arising from the palate.

2.1 Etiology

Melanomas are categorized into four different types according to anatomic region and pathological factors: 1. melanomas arising from skin without chronic sun damage, 2. melanomas on skin with chronic sun damage, 3. acral melanomas, and 4. mucosal melanomas (Curtin et al., 2006). The etiology of primary oral malignant melanoma is still unknown. Cigarette smoking, denture irritation and alcohol have been mentioned as possible risk factors, but evidence remains obscure. Intraoral malignant melanomas arise from melanocytic cells of the oral cavity, which represent a minority of all cells of the mucosal membrane. Although just a few melanocytes are present in the oral mucosa the potential to develop malignant melanomas clearly exist. Indeed it is recognized that malignant melanoma can very rarely develop in almost any organ suggesting that circulating melanocytes may be responsible. About 5-30 % of primary malignant melanomas are preceded by oral pigmentations for months or even years. It has been suggested that melanosis represents the initial phase characterized as the radial phase of growth and precedes the invasion of underlying tissues (vertical growth) by years, however the histological spectrum of benign pigmented lesions is wide and a pre-existing pigmented lesion is not usually associated with mucosal melanomas. Most arise as new lesions, from apparently healthy mucosa.

Malignant melanomas develop from melanocytes derived from the neural crest. The most frequently primary sites in the oral cavity are the palate and maxillary gingiva (Barker et al., 1997, Hicks and Flaitz, 2000, Lee et al., 1994). Mucosal melanomas are considered to be more aggressive tumors compared with cutaneous melanomas and they are more inclined to metastasize into regional and distant sites, recur locally or regionally resulting in a high rate of disease specific-death.

2.2 Epidemiology

Malignant melanoma in the head and neck area is rare. Oral cavity primary malignant melanoma comprises 6.3 % of all melanomas in the head and neck area and only about 0.7 – 1.6 % of all melanomas arise in the oral mucosa (Chang et al., 1998, Moore and Martin, 1955). The incidence of primary mucosal melanomas of the head and neck is approximately four per 10 million population per year (Hicks and Flaitz, 2000). The most frequently affected oral sites are the palate and maxillary gingiva (Barker et al., 1997, Hicks and Flaitz, 2000, Lee et al., 1994). The incidence in Japan is much higher than in western countries, in which it occurs with less than 1% (Batsakis, 1982, Brandwein et al., 1997, Umeda and Shimada, 1994). The age of the patients varies between 20 and 80 years, the mean age reported in the literature ranges from 56 to 66.5 years (Gorsky and Epstein, 1998, Hicks and Flaitz, 2000, Patel et al., 2002, Rapidis et al., 2003, Rapini et al., 1985) and a modest male preponderance has been described (Rapini et al., 1985).

2.3 Prognosis

The prognosis is adverse. Due to delayed diagnosis early invasion of surrounding tissue, local lymph node involvement and distant metastasis, the prognosis of oral mucosal melanomas is very poor. The reported 5-year survival rates vary in the literature between 6 and 18 % but also between 45 and 48 % (Enroth CM, 1975, Lee et al., 1994, Lund, 1993, Ravid and Esteves, 1960, Stern and Guillaumondegui, 1991). Gingival melanoma has a slightly better 5-year survival rate (18%) than that of palatal melanoma (11%), with a longer median survival period (46 months vs. 22 months). These wide variations are probably due to the fact that comparable classifications are still not agreed internationally. The routine use of tumor thickness of mucosal melanomas, as described by the Breslow or the Clark levels are not widely performed as a routine method in everyday experience (Prasad et al., 2003, Prasad et al., 2002, Thompson et al., 2003). Therefore an evidence base derived from histopathological assessment and subsequent prognosis for malignant mucosal melanomas is lacking. Despite this many reports show a correlation between tumor thickness and prognosis (Mücke et al., 2009, Prasad et al., 2004). Many authors agreed that the survival rate was not only closely related to the stage of the tumor, but the treatment that patients received (Mücke et al., 2009, Prasad et al., 2003, Prasad et al., 2004, Rapidis et al., 2003, Rapini et al., 1985, Tanaka et al., 2004, Temam et al., 2005).

The vast majority of the head and neck mucosal melanomas are Stage 1 at the time of presentation, Prasad et al defined a 3-level-microstaging system, which represents different microanatomical compartments separated by tissue barriers. They found out that this microstaging system is prognostically significant and an independent predictor concerning the 5-year survival rate and the recurrence-free survival (Mücke et al., 2009, Prasad et al., 2004). In 2009, the American Joint Committee on Cancer (AJCC) Melanoma Staging Committee used previously published guidelines and determined criteria which were used

in the TNM classification and the stage groupings (Balch et al., 2009). These criteria are established for all kinds of melanomas and are proposed as follows:

1. In patients with localized melanoma, tumor thickness, mitotic rate, tumor burden, and ulceration were the most important prognostic factors.
2. The mitotic rate indicates the level of invasion as a primary criterion for defining T1b melanomas.
3. Components that defined the N category were the presence and number of metastatic nodes of the primary melanoma.
4. All patients with microscopic nodal metastases, regardless of extent of tumor burden, are classified as stage III.
5. On the basis of a multivariate analysis of patients with distant metastases, the two dominant components in defining the M category continue to be the site of distant metastases and an elevated serum lactate dehydrogenase level.

Factors that have been associated with worse disease-specific survival include clinical stage at presentation, thickness of the tumor, tumor burden at the time of staging (microscopic vs. macroscopic), presence or absence of primary tumor ulceration, presence of vascular invasion, melanosis, and development of nodal and distant metastasis ($p < 0.001$) (Balch et al., 2009). Multiple local recurrences are the most common cause of treatment failure and may occur 10-15 years after primary treatment. The most common sites of distant metastases include the lungs, brain, liver and bones.

2.4 Staging

The criteria to verify the presence of a primary intraoral melanoma are:

1. demonstration of clinical and microscopic tumor in the oral mucosa
2. presence of junctional activity in the lesion
3. inability to show any other primary site.

All patients have to fulfill all these criteria to verify the diagnosis of primary oral malignant melanoma (Greene et al., 1953). In addition to the standard staging procedure, patients should be observed by means of: sonography, gastroscopy and bronchoscopy in order to exclude other potential primary sites and confirm by exclusion the diagnosis of primary oral mucosal malignant melanoma (Mücke et al., 2009). It is important to confirm the comparatively rare finding of primary oral mucosal malignant melanoma is supported by exclusion of other potential mucosal sites for example the respiratory and the gastrointestinal tract, which are more frequently found than oral melanomas (Lee et al., 1994, Mendenhall et al., 2005, Prasad et al., 2003).

Patients should be staged according the TNM Melanoma Staging System of the American Joint Committee on Cancer which includes in the staging the extent of the tumor status as well as the extent of the nodal status, but does not provide a specific guideline for oral mucosal melanomas. Therefore a simplified staging system has been established, which classifies tumors in 3 stages: Stage I to localized disease, confined to the primary site, stage II the primary lesion with cervical lymph node metastasis and stage III for distant metastasis.

As previously described there is a 3-level-microstaging system which relates to the prognostic outcome. In this system each level represents a microanatomical compartment, which is defined through tissue barriers. Breach of each barrier correlates with a progressively worse survival rate. Level I is defined as an in situ mucosal melanoma or microinvasion, Level II as an invasion limited to lamina propria and Level III is defined as melanoma with a deep invasion into surrounding tissue such as bone cartilage or skeletal muscles.

2.5 Histopathology

The surface architecture from oral melanomas ranges from macular to ulcerated and nodular. As recommended at the WESTOP (Western Society of Teachers of Oral Pathology) Banff Workshop, oral malignant melanomas should be separately considered from the cutaneous forms and proposed to subclassify them according to the histological pattern into: in situ melanoma, invasive, combined (invasive melanoma with in situ components) and atypical melanocytic proliferation (in case where diagnosis is equivocal) (Barker et al., 1997). In situ melanomas are limited to the epithelium and the epithelial-connective tissue interface, and represent 15% of the oral melanomas. They show a proliferation of atypical melanocytes characterized through hyperchromatic and angular nuclei with infrequent mitotic activity. The melanocytes may be arranged irregularly at the epithel connective tissue-interface or may be distributed in aggregates.

Invasive pattern in which the melanoma extend into the connective tissue is represented in 30% of oral melanoma, showing a wide range of cell types including spindle, plasmocytoid, clear cells and epithelioid, arranged into sheets or organoid/alveolar formation. The large and vesicular nuclei appear frequently with prominent nucleoli and rare mitosis. 55% of oral melanomas having combined pattern, which is typical for advanced lesions (Barker *et al.* 1997, Femiano *et al.* 2008).

In most instances melanomas contain melanin pigmented tumor cells but amelanotic melanoma show a lack of melanin production, which exacerbates the correct diagnosis, because amelanotic melanoma can mimic a variety of poorly differentiated carcinoma or cell lymphoma. For distinguishing these melanomas from other tumors immunohistochemical stains have been proven to be helpful. For differential diagnosis, it should be claimed that immunohistochemical staining of the following markers is mandatory and the basis of the diagnosis of an oral malignant melanoma.

Immunohistochemical markers include S-100 protein, gp100 (HMB-45) and Mart-1 (Melan-A) (Messina et al., 1999). These markers are also used for the identification of micrometastases in lymph nodes (Messina et al., 1999).

The Antibody HMB-45 reacts with the melanosomal glycoprotein gp100, showing a positive staining in active early melanosome formation and showing epithelioid lesions intensely immunoreactive for HMB-45. It is considered as more specific but less sensitive than the S-100 protein, an acidic calcium binding protein, which is a very sensitive marker for nevus and melanoma cells, and even spindled lesions appear intensely immunoreactive for S-100 protein (Blessing et al., 1998, Gazit and Daniels, 1994). Melan-A is considered to be specific for melanoma cell lines, as a product of the MART-1 gene it is a melanocytic differentiation marker which is recognized on melanomas as an antigenic target of T lymphocytes (Kawakami et al., 1994).

In recent years some molecular markers, like the Ki67 antigen emerged as potentially prognostical indicators, however the role of potential therapeutic relevance of KIT inhibitors in mucosal melanoma further needs to be investigated.

2.6 Therapy

Regarding the treatment of the four types of melanomas, melanomas arising from skin without chronic sun damage, melanomas on skin with chronic sun damage, acral melanomas and mucosal melanomas, most authors agree that radical ablative surgery with wide local excision of the primary lesion and dissection of metastatic lymph nodes are the basis of every curative therapy (Curtin et al., 2006). Controversial points are the margin of

the excision, the optimal time to dissect the local lymph nodes, and the uncertainty of the extent of lymph nodes dissection. Surgery is the mainstay of treatment but can only be accomplished if vital parts of the body are not affected and therefore anatomical limitations often make a radical excision impossible.

The following protocol refers to the extent of margins:

1. Excision of the primary lesion including at least 1 – 2 cm of healthy tissue based on the primary tumor extent and thickness
2. Lymph node dissection and removal of lymph node metastases
3. Consideration of radiochemotherapy (limited evidence as to the benefit of postoperative radiotherapy exists for other anatomical sites).

2.6.1 Surgery

Principles defined by the first tumor resection in 1857 remain viable today in that the treatment of all melanomas should be performed by wide resection. Cutaneous melanomas are still treated by that principle to avoid local recurrence (Essner, 2003, Hauschild et al., 2003, Veronesi and Cascinelli, 1979). Limited excision of the primary lesion as well as excisional and incisional biopsies are associated with an increased risk of causing accidental dissemination of malignant cells within the adjacent tissues or even into the blood or lymphatic stream with probable devastating consequences (Harter et al., 1992, Kusakawa et al., 2000).

The surgical margin also depends on the thickness of the tumor. In patients with tumors less than 1 mm thickness, a surgical margin of 1 cm has been shown to be adequate, compared with wide tumor margin resections of 3 cm (Veronesi et al., 1988). None of these patients developed local recurrence in the follow-up period revealing an adequate resection extent. In patients with tumor thickness of 1 to 2mm local recurrences were found in both study groups of patients receiving resection margins of 1 cm or 3 cm without significant differences, although more recurrences were found in the 1 cm group (Veronesi et al., 1988). The evidence, that resections of 1 to 2 cm around the tumor are sufficient and show similar results in local control and overall survival, have been also found by other studies comparing the long term results of different resection protocols in varying tumor extent and thicknesses (Balch et al., 1993, Heaton et al., 1998). The local recurrence rate in these studies is 1.7% with 2 cm surgical margin compared with 0.8% with 4 cm after six years without statistically significant differences (Balch et al., 1993, Heaton et al., 1998). The surgical therapy within the oral cavity remains problematic because wide resection margins require also reconstruction techniques after tumor ablation to avoid mutilation, functional impairment and a poor quality of life (Mücke et al., 2009, Mücke et al., 2011, Mücke et al., 2010). In some instances the suggested resection margins would compromise vital structures which mean a compromise between theoretically ideal margins and postoperative function and quality of life must be made.

Regional lymph nodes are the most common sites of metastases for all melanomas. Palpable lymph nodes in the neck or fixed to the adjacent tissues should be suspicious for metastases. Radiologically, lymph nodes larger than 1 cm must also be considered to be involved by metastases. There exists a correlation between the tumor thickness and the occurrence of regional lymph node metastases. Primary lesions less than 1 mm thickness are considered to yield a rate of < 10% lymph node metastases, 1.01 to 2.00 mm are accounting for about 20%, 2.01 to 4.00 mm are accounting for 33%, and >4.00 mm are associated with a risk of

lymph node involvement of more than 40% at the time of staging (Morton et al., 2005, Morton et al., 2003, Morton et al., 1993). Another study evaluated an exponential increasing of lymph node metastases if the tumor becomes thicker. A thickness of 0.76 to 1.50 mm was associated with regional lymph node metastases in 2 to 25% of cases. In tumors with a thickness between 1.51 to 4.00 mm the rate of regional lymph node metastases developed to 57%. A tumor thickness larger than 4 mm was associated with microscopic presence of metastases within the lymph nodes (Balch, 1999, Balch et al., 2000, Balch et al., 1996). There has been evidence that lymph node dissection resulted in an increase of overall survival compared to patients receiving palliative treatment to the neck only (Balch, 1999, Balch et al., 2000, Balch et al., 1996, Balch et al., 1993, Cascinelli et al., 1998). Negative lymph nodes are a strong prognostic factor for survival, whereas lymph node metastases yield a 6 times higher relative risk for death (Balch et al., 2009, Gershenwald et al., 1999).

Patients who suffered from oral mucosal malignant melanomas are often diagnosed at an advanced stage followed by ulceration, microsatellites or regional nodal metastases (Chaudhry et al., 1958, Morton et al., 1993, Mücke et al., 2009, Patel et al., 2002, Prasad et al., 2004, Rapidis et al., 2003, Temam et al., 2005). This high rate of regional lymph node metastases means that patients at risk should be considered for therapeutic elective neck dissection with a low threshold for surgery. Although no randomized trials on the treatment of the regional lymph nodes for oral mucosal malignant melanoma exist due to the rarity of this tumor, there is little doubt about this treatment approach. (Balch et al., 2009, Balch et al., 1996, Cascinelli et al., 1998, Chaudhry et al., 1958, Essner, 2003, Hauschild et al., 2003, Medina et al., 2003, Morton et al., 2003, Morton et al., 1993, Mücke et al., 2009, Patel et al., 2002, Tanaka et al., 2004, Temam et al., 2005). As the oral melanoma is very aggressive and the texture of the mucosa is different from the cutis with an earlier occurrence of lymph node metastases, there is actually no role for the sentinel lymph node technique, as the risk of micrometastases has to be excluded by standard therapeutic neck dissection (Prasad et al., 2004, Snow and van der Waal, 1986, Umeda and Shimada, 1994).

2.6.2 Radiotherapy

Radiotherapy has been used to control local recurrence (Balch et al., 1993, Harwood, 1983, Schmidt-Ullrich and Johnson, 1996, Storper et al., 1993). Postoperative, adjuvant radiation therapy results in a lower local recurrence rate in comparison to patients without radiotherapy treated by wide surgical resection only (Harwood, 1983). Hyperfractionated accelerated radiotherapies have appeared most useful (Harwood, 1983, Schmidt-Ullrich and Johnson, 1996, Storper et al., 1993), although the differences were not found to be statistically significant. The adjuvant performance of radiotherapy should be considered in advanced stage malignant melanomas of the oral cavity. There are still no data available from randomized trials regarding the efficacy of adjuvant radiotherapy.

2.7 Adjuvant therapy

Adjuvant therapy modalities vary and include the application of non-specific immunostimulants (Grooms et al., 1977, Morton et al., 1970, Morton et al., 1974, Sondak and Wolfe, 1997), specific immunostimulants (Interferons), vaccination therapy, cytotoxic chemotherapy, and target-therapy.

Non-specific immunostimulants like Bacille Calmette-Guérin (BCG), *Cryptosporidium parvum*, Levanisole, thymosin, isoprinosine, transfer factor and retinoids, and interleukin

had been applied by local injection into the tumor (Grooms et al., 1977, Morton et al., 1970, Morton et al., 1974, Sondak and Wolfe, 1997). Although some authors reported limited evidence that survival was increased by this therapy this adjuvant therapy failed to prove an advantage in comparison with control treatment of any kind. Most studies performed were retrospective studies (Grooms et al., 1977, Morton et al., 1970, Morton et al., 1974, Sondak and Wolfe, 1997).

After non-specific immunostimulants have failed to establish an effect on overall survival or an improvement of symptoms, attention was paid to more specific immunostimulants such as Interferons. These glycoproteins showed an immunomodulatory and anti-tumoral effect, which was thought to be beneficial in melanoma patients. Expression of major histocompatibility complex molecules, enhancing of antigen presentation by dendritic cells, inhibition of angiogenesis, and a directly antiproliferative effect combined with the expression of melanoma-specific antigen and the stimulation of natural killer cells directly affecting the tumor was a promising treatment modality (Ascierto and Kirkwood, 2008, Janku and Kurzrock, 2010, Jonasch and Haluska, 2001, Jonasch et al., 2000, Kalani et al., 2008, Pfeffer et al., 1998). Unfortunately early enthusiasm has waned and this strategy is more and more critically debated (Janku and Kurzrock, 2010). The effect of interferon- α (IFN- α) has been well evaluated in different clinical trials in melanoma patients and a dose-dependent and duration-dependent effect has been found, especially in high-risk patients with the presence of lymph node metastases or thick melanoma patients (Kirkwood et al., 2001, Kirkwood et al., 1996, Punt and Eggermont, 2001). In contrast, high-dose IFN- α is often associated with severe toxicity (Eggermont, 2002, Eggermont and Gore, 2002), and the outcome reported by the Southwest Oncology Group revealed no clinical benefit for patients who received adjuvant therapy. Indeed they may have done worse when compared to the patients who received no adjuvant therapy (Barth and Morton, 1995, Taylor et al., 1992).

Another adjuvant method for the therapy of melanoma patients are vaccination therapies. Based on the evidence that the immune system plays a natural role in melanoma progression in the same way that the previous treatment modalities tried to act at the same mechanism, vaccination therapy is designed to activate the host immune response to tumor-associated antigens. In the lack of information about specific tumor antigens, the tumor cell was the best source of antigens for activating the immune system (Hersey et al., 2002, Hersey et al., 1987, Sun et al., 1999). Previous methods of producing vaccines are always based on tumor cell preparations, but there is still no role for vaccination therapies in oral malignant melanomas.

The most established therapy beside surgery combined with radiotherapy remains cytotoxic chemotherapy. Multiple trials about a variety of cytotoxic drugs in adjuvant treatment have been performed but no study demonstrated benefits of adjuvant chemotherapy in melanoma patients at high risk for relapse or affecting overall survival significantly in controlled randomized trials. The mainstay of chemotherapy remains the palliative situation. The mainstays of cytotoxic chemotherapy include; dacarbazine, the nitrosoureas, the vinca alkaloids, cisplatin, paclitaxel, and bleomycin. The use of additional chemotherapeutic agents is still evolving. Single-drug therapy with dacarbazine reported an objective response in 18-22% of patients with measurable metastatic disease (Hill et al., 1979, Hill et al., 1981, Houghton et al., 2006, Houghton et al., 1996, Lee et al., 1995). Some trials describe efficacy of dacarbazine as a postsurgical adjuvant, but suggested no significant clinical benefit in the treatment of patients with high risk melanoma (Hill et al., 1979, Hill et al., 1981, Veronesi et al., 1982). Multiagent cytotoxic therapy is similarly unhelpful in the

adjuvant setting. Other trials of multiagent chemotherapy using nitrosoureas, dactinomycin and vincristine present contrary results (1983, Castel et al., 1991, McClay et al., 2000, Pawlik and Sondak, 2003, Wood et al., 1978).

3. Conclusion

The incidence of oral melanoma is very low and results of the treatment are still poor partly due to the advanced stage of tumor at presentation. No single management strategy or guideline can be considered the standard of care on the basis of current data. Within the head and neck the extent of the tumor, spread to the regional lymph nodes, systemic disease and histopathological variables have to be integrated into the disease staging and related to the patients' co-morbidities and personal aims. Radicality of resection has to be balanced against the feasibility of reconstruction of the resected area obtaining form and function as well as the quality of life the patient requires. Treatment of oral mucosal malignant melanoma may not be entirely consistent with the treatment of cutaneous malignant melanoma. The thickness of the primary lesion, stage of regional lymph nodes, sex, age, the reaction of the lesion to treatment are important factors influencing prognosis and treatment choices of the disease. To date, due to the rarity of this tumor entity, no randomized trials exist demonstrating any optimal treatment algorithm.

Multicenter studies collecting data about the treatment strategies and outcomes of patients suffering from oral malignant melanomas are necessary to identify the best treatment algorithm based on patient related clinical work. Oral melanomas are different from cutaneous melanomas and such studies would provide us a far better insight into their behaviour. The tumor thickness is a variable that most accurately determines therapy and prognoses, therefore, the extent of surgery margins should be decided based on the invasive depth of the primary lesion, the neck addressed on the basis of imaging staging but with a low threshold for intervention. Reconstruction follows conventional approaches but adjuvant therapy is currently disappointing.

An increasing understanding of tumour immunology and biology has led to innovative therapies which are necessary if an effective treatment for oral malignant melanomas is to be developed in the future. The application of new or established technologies in experimental tumor models is necessary to increase the potential for such treatments (e.g. proteomics, targeted therapy, vaccination therapy), but is currently still an area of experimentation and hope rather than pragmatic clinical practice.

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Choroidal Melanoma

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1. Introduction

Uveal melanoma is the most common primary ocular malignancy in adults. There are about 0.6-0.8 new patients per 100,000 inhabitants (in Europe). Several studies have demonstrated that complete removal of the tumour by enucleation is not advantageous compared with conservative therapies in terms of metastatic spread and survival.

The primary treatment for uveal melanoma depends on many factors: size, location, extraocular extension, visual function, age and performance status.

2. Enucleation

Enucleation has been the standard treatment since the nineteenth century and is still the treatment of choice in large uveal melanomas.

Enucleation is still required in a significant proportion of patients, either because the tumour is too extensive at presentation or because of the complications of conservative therapy. The procedure is performed in the surgeon's preferred fashion. It is essential to perform binocular indirect ophthalmoscopy after draping the patient to confirm that the correct eye is being removed. The main complication specific to uveal melanoma is orbital tumour recurrence, which is rare. If this occurs, it is treated with local resection and external beam radiotherapy. It was previously believed that surgical manipulations during enucleation disseminated tumour cells into the vascular system, thereby causing metastatic disease; however, as we will see later, COMS-10 study has shown no improvement of survival after pre-enucleation radiotherapy, thereby casting doubt on this hypothesis.

Globe preserving techniques include: laser photocoagulation, transpupillary thermotherapy, local resection, radiotherapy and gammaknife.

Observation without treatment is a modality used for melanomas that are small and have dormant characteristics. Patients managed by this strategy are often asymptomatic and have the lesion picked up on routine ocular examination. Another subgroup of patients who may be managed by observation includes elderly patients with severe systemic health problems or short life expectancy.

Radiotherapy (RT) offers a conservative treatment for those patients who are suitable for visual and motion conservation.

3. Radiobiology

Radiations kills a tumour either by producing free radicals that destroy cellular DNA immediately or by induction of mutations that go on to kill tumour cells over a protracted

period of time. Radiation also induces vascular fibrosis and secondary hypoxia, which again may take time to cause cell death. Thus, RT provides both short- and long-term effects¹. There are two main modalities: brachytherapy and external beam radiotherapy (that includes charged particles and special techniques like intensity modulated radiotherapy).

4. Brachytherapy

In the United States, controversies about the appropriate management of choroidal melanoma led to the development of the COMS (Collaborative Ocular Melanoma Study) group, which conducted a series of randomised studies on the use of enucleation, brachytherapy and radiotherapy. To date, their principal results have been that preoperative radiotherapy for large size melanomas does not improve survival compared with enucleation alone, and that there is no difference as regards survival between those patients treated with I-125 seed brachytherapy and those who underwent enucleation. The results of these studies show brachytherapy as an alternative to enucleation.

Various materials for delivering radiation have been investigated in brachytherapy. Iodine-125 is currently the most commonly used isotope for plaque radiotherapy of choroidal melanoma. Although cobalt-60, ruthenium-106, Iridium -196, strontium-90 and palladium-103 have also been used.

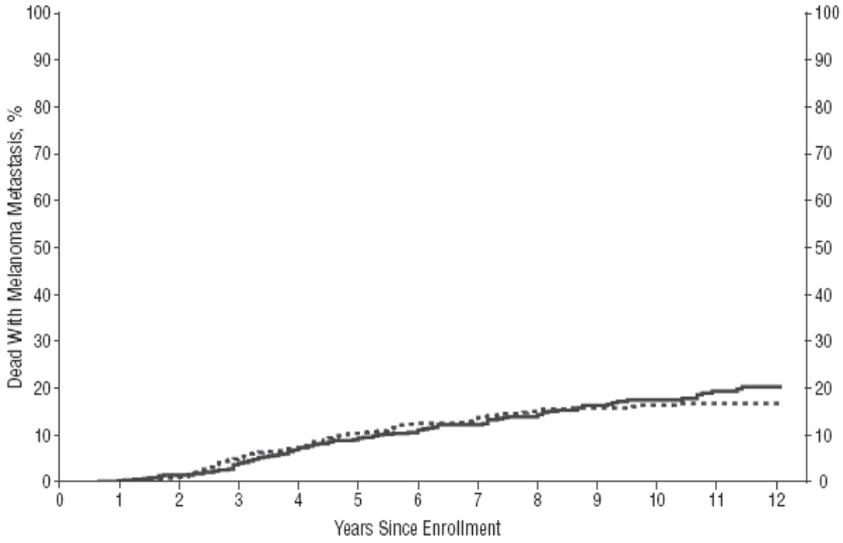
Radionuclide	Symbol	Type	Energy	Half-life	Introduced	depth
Cobalt	Co-60	Gamma/beta	1.3MeV/320Kev	5.2 years	1948	
Ruthenium	Ru-106	Beta	293 KeV	373 days	1964	5 mm
Iodine	I-125	Gamma	27-35 KeV	60 days	1975	
Strontium	Sr-90	Beta	546 KeV	29 years	1983	5 mm
Iridium	Ir-192	Gamma/Beta	600KeV/370 KeV	74 days	1983	
Palladium	Pd-102	Gamma	21 KeV	17 days	1986	

Table 1.

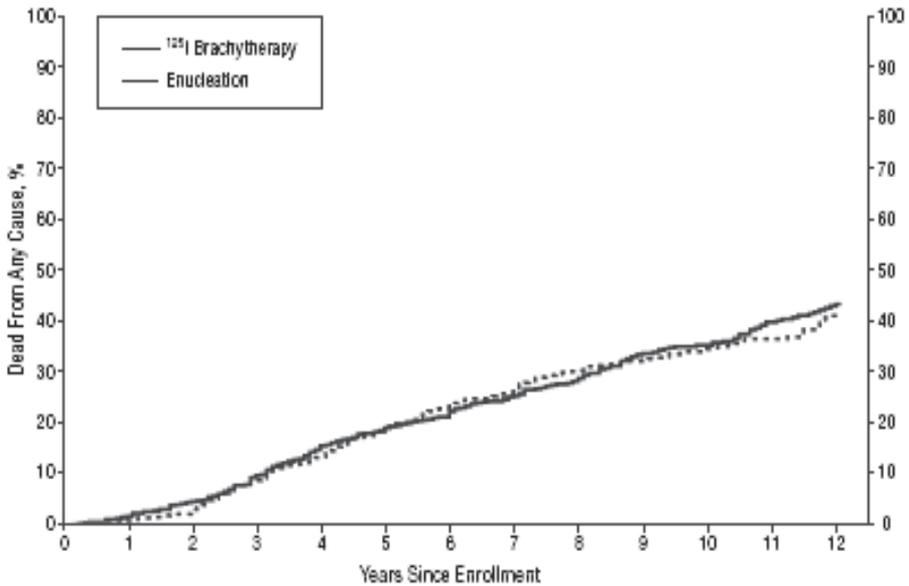
Before the COMS trials were initiated in 1986, interest in radiation therapy had increased because of the potential for saving the eye and perhaps some vision.

The COMS-18 trial enrolled 1317 patients with medium size melanomas to enucleation or brachytherapy with iodine-125 (I-125) plaques. Six hundred and sixty were randomly assigned to enucleation and 657 to I-125 brachytherapy (85 Gy). Only 2 patients in the enucleation arm were found to have been misdiagnosed when histopathology was centrally reviewed. All but 17 patients (1.3%) received the assigned treatment. Adherence to the brachytherapy protocol was excellent, with 91% of patients treated per protocol. Based on time since enrolment, 1072 patients (81%) had been followed for mortality at 5 years and 416 (32%) at 10 years. A total of 364 patients had died: 188 (28%) of 660 patients in the enucleation arm and 176 (27%) of 657 patients in the brachytherapy arm. The unadjusted estimated 5-year survival rates were 81% and 82%, respectively; there was no clinically or statistically significant difference in survival rates overall ($P = 0.48$, log-rank test). The

adjusted estimated risk ratio for I-125 brachytherapy vs enucleation was 0.99 (95% confidence interval [CI], 0.80-1.22). Five-year rates of death with histopathologically confirmed melanoma metastases were 11% and 9% following enucleation and brachytherapy respectively; after adjustment, the estimated risk ratio was 0.91 (95% CI, 0.66-1.24). Graphs 1 and 2. They concluded that mortality rates following I-125 brachytherapy did not differ from mortality rates following enucleation for up to 12 years after treatment ².



Graph 1. Dead with melanoma metastasis at COMS-18 trial



Graph 2. Dead from any cause at COMS-18 trial

Visual acuity declined in a substantial proportion of eyes in the brachytherapy group. There was a quadrupling of the minimum angle of resolution, or loss of 6 or more lines of visual acuity from baseline in 18% of patients at 1 year, 34% at 2 years and 49% at 3 years. The risk of vision loss was associated with a history of diabetes, thick tumours, tumours close to or beneath the macula, tumours with secondary retinal detachments and tumours that were not dome-shaped³. Secondary strabismus has also occurred in 5% of patients as a result of moving extraocular muscles in order to properly place the plaque to hold the radioactive seeds (see figure 2).

Tumours with larger basal diameters are more likely to recur. Karlsson et al. found that patients with local tumour recurrence, which is likely to occur at the tumour margin, were at greater risk of life-threatening distant metastasis, having a 5-year survival rate of 58% compared with 82% for those without local recurrence. However, while local control of choroidal melanomas treated with brachytherapy has been reported at more than 90%, many treated eyes develop complications secondary to radiation delivered to adjacent structures. Radiation retinopathy has been identified in up to 43%. Other complications include optic atrophy, cystoid macular edema, cataracts, glaucoma, central retina vein occlusion and scleral necrosis.

In the COMS-10 trial of preoperative radiation, patients with large tumours were randomized to enucleation alone or to enucleation preceded by 20 Gy of external beam radiation. This trial was designed to see if radiation before enucleation (removal of the eye) would prevent metastasis. The idea was to see if pre-operative irradiation would sterilize any cells that might break free during surgery.

The two randomly assigned groups of patients were followed for at least five years or until death and were compared on the basis of length of remaining life and other outcomes. A total of 1,003 patients were enrolled; 506 were assigned to enucleation alone and 497 to pre-enucleation radiation. With 5-year outcome known for 801 patients enrolled (80%), the estimated 5-year survival rates and 95% confidence intervals (CIs) were 57% (95% CI, 52% to 62%) for enucleation alone and 62% (95% CI, 57% to 66%) for pre-enucleation radiation. Among the baseline covariates evaluated, only age and longest basal diameter of the melanoma affected the prognosis for survival to a statistically significant degree. The risk of death among patients treated with pre-enucleation radiation relative to those treated with enucleation alone after adjustment for baseline characteristics of patients, eyes, and tumours was 1.03 (95% CI, 0.85 to 1.25). Of 435 deaths classified by the Mortality Coding Committee, 269 patients had histologically confirmed melanoma metastases at the time of death. Estimated 5-year survival rates for this secondary outcome were 72% (95% CI, 68% to 76%) for enucleation alone and 74% (95% CI, 69% to 78%) for pre-enucleation radiation. The Large-sized Choroidal Melanoma Study concluded that patients who received external irradiation to their eye before it was removed, had an equal chance of developing metastatic disease as compared to those who were treated by enucleation (removal of the eye) alone.

This study did not find any survival difference attributable to pre-enucleation radiation of large choroidal melanoma, using the COMS fractionation Schedule⁴.

Accrual to a nonrandomized pilot study to assess the feasibility of a randomized trial for small tumors was halted in 1989. Additional follow-up of these patients was carried out from 1994 to 1996. From December 1986 to August 1989, 204 patients with small choroidal melanoma, not large enough to be eligible for the COMS clinical trials, were offered

participation in a nonrandomized prospective follow-up study. Small choroidal melanomas were defined as 1.0 to 3.0 mm in apical height and at least 5.0 mm in basal diameter. A total of 204 patients were enrolled in the study. Patients were followed up annually through August 1989. Two additional assessments of treatment status and mortality were conducted in 1993-1994 and 1995-1996. The median length of follow-up was 92 months. Eight percent of patients were treated at the time of study enrolment and an additional 33% were treated during follow-up. Twenty-seven patients have died; 6 deaths were reported by the clinical center as due to metastatic melanoma. The Kaplan-Meier estimate of 5-year all-cause mortality was 6.0% (95% confidence interval, 2.7%-9.3%) and 8-year all-cause mortality was 14.9% (95% confidence interval, 9.6%-20.2%).

The study concluded that healthy patients, average age of 60 years, without a previous diagnosis of malignant disease who had small choroidal lesions judged to be melanoma had a low risk of dying within 5 years⁵.

5. Implant sequence

The procedure is performed thanks to the joint efforts of a multidisciplinary team composed of ophthalmologists, radiation oncologists, anaesthetists, physicists, and specialised radiotherapy technicians.

Patient preparation:

1. Placing of the radioactive plaque. This is carried out with both local and general anaesthetic, depending on each case, but mostly using retrobulbar anaesthesia with akinesia.
2. Gestures: In general, a limbic peritomy is performed, dissecting Tenon's capsule to leave the scleral surface which coincides with the tumour base exposed. If the lesion affects the muscle insertion area, the muscle must be disinserted.
3. CTV (Clinical target volume) Determination: Pupilar transillumination is used for the localization and marking of the base of the melanoma. Various non-absorbable sutures are placed at the scleral level, at positions marked by an inactive phantom, in order to check the correct size of the proposed plaque; the definitive radioactive plaque is then positioned and the sutures are tied. The diameter of the radioactive applicator should be 2 mm greater than the tumour base, to ensure that the microscopic disease is covered by a safety zone for complete irradiation of its margins. Additional margins are not taken to cover eye movements.
4. Post-operative: Anti-inflammatory drugs and antibiotics are administered subconjunctivally, topically and systemically in theatre. The patient is subsequently transferred to a darkened room.
5. Treatment course: The treatment is given as scheduled and all patients remain admitted to the Brachytherapy Unit. During admission, analgesic and prophylactic antibiotic treatment is prescribed.
6. 6. Implant removal: On extraction of the ophthalmic plaque, the patient is again transferred to the implant room where it is disinserted under anaesthesia and complete removal of the radioactive load is checked. Once the plaque had been extracted, the patient returns to his room where he remains until recovery and discharge. See figure 1, 2, 3 and 4.

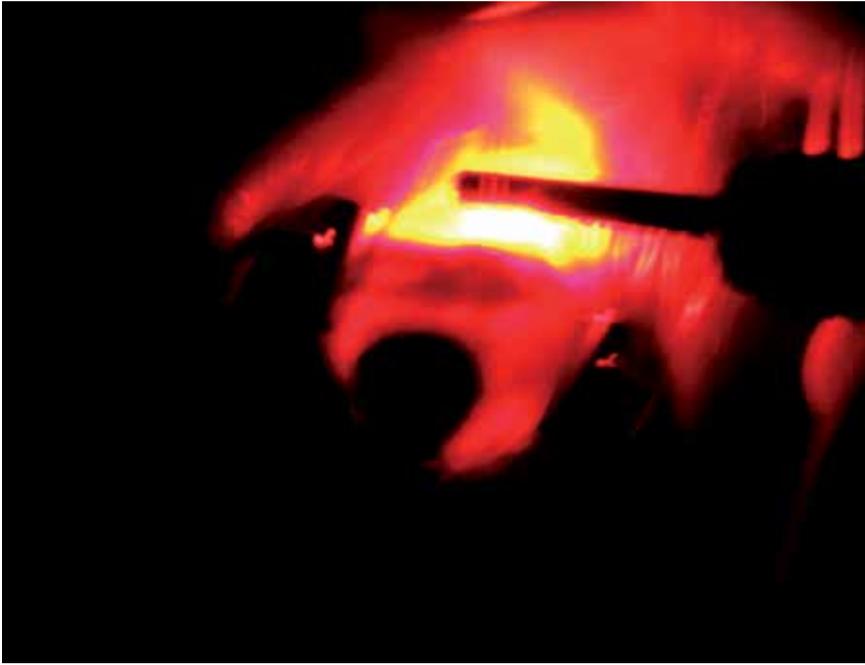


Fig. 1. Pupilar transillumination

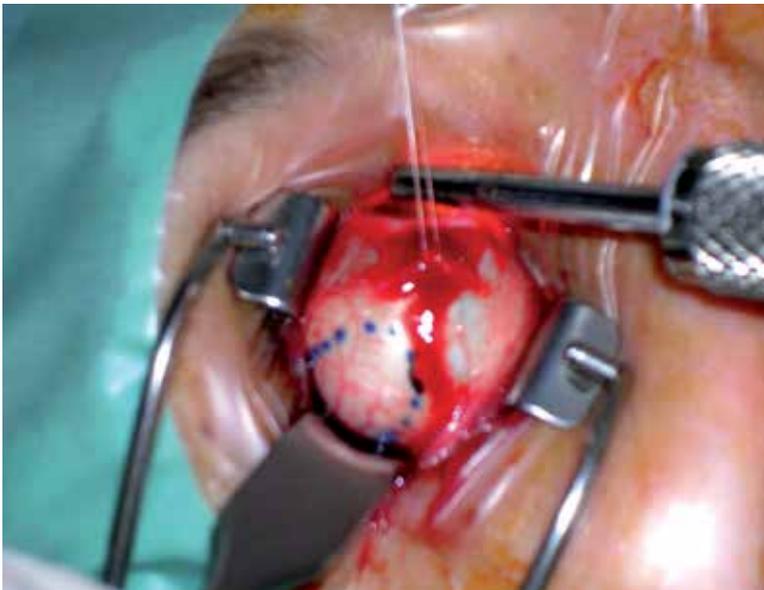


Fig. 2. Target volume delineation

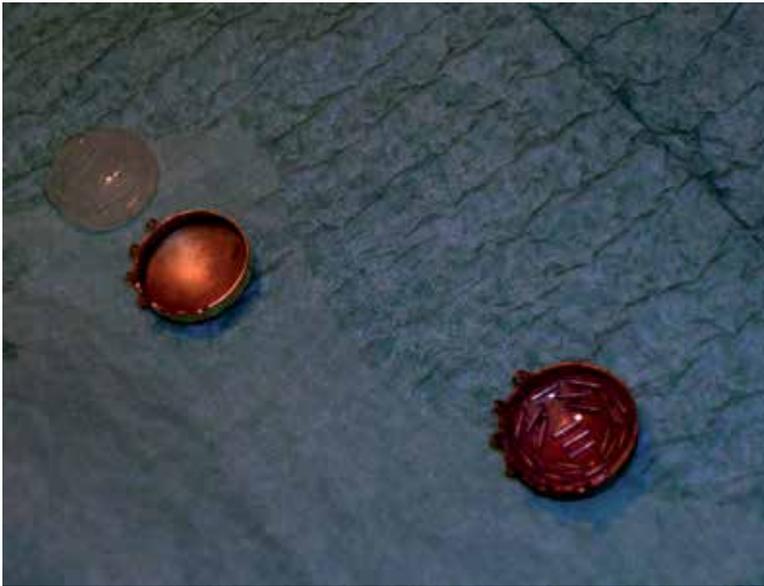


Fig. 3. Plaque with I-125 seeds.



Fig. 4. Eye with the plaque in place.

6. Prescription dose

The prescription point is defined as the tumour apex for tumours measuring 5.0 mm or more in apical height; but for smaller tumours, the prescription point is 5 mm from the interior surface of the sclera. In 1996, the COMS adopted the recommendations of the American Association of Physicists in Medicine Task Group (TG-43) regarding dosimetry of interstitial sources in anticipation of a revised calibration standard for I-125 seeds by the

National Institute for Standards and Technology (NIST). Under the TG-43 formalism, the absorbed dose at the prescription point was 85 Gy delivered at a rate of at least 0.42 Gy/hour, but no more than 1.05 Gy/hour. Before adopting the TG-43 formalism, the prescribed total dose was calculated to be 100 Gy; however, the actual amount of radiation delivered was not affected by the change. All dose data included in the majority of reports were recalculated based on the TG-43 formalism. The actual radiation dose delivered to the prescription point, tumour apex, sclera at the center of the plaque and critical structures within the eye was reported to and confirmed by the COMS Radiologic Physics Center (Houston, TX). Doses were calculated based on presumed plaque location, I-125 seed activity and location in the plaque, and times of plaque insertion and removal.

7. Combined treatment

Combined plaque irradiation and laser photocoagulation or thermotherapy have been used recently to increase the likelihood of complete local tumour destruction, particularly in patients with tumours adjacent to the optic disc. In 1998, Shields et al. published the results of 100 patients treated with plaque and transpupillary thermotherapy and found a recurrence rate of 3% in 8 years.

8. Proton beam radiotherapy

Proton-beam radiotherapy (PBRT) was first utilized in the management of uveal melanoma in 1975⁶. It is now predominantly used for choroidal and ciliary body melanomas. PBRT is an alternative method of delivering radiation to an ocular tumour that uses charged particles, either protons or helium ions (PBRT). Tantalum clips are fixed to the episcleral surface around the base of the tumour, and charged particles are then directed toward the tumour from an anterior approach. With this technique the dose is delivered in four or five equivalent fractions over a 7-days period. Typically a total dose of 70 Cobalt gray equivalent (CGE) is administered over 5 fractions.

The density of ionization of protons increases markedly near the end of their path (Bragg peak). This characteristic enables accurate treatment, especially important for large lesions close to vital ocular structures. The advantages of charged particles include a uniform dose distribution throughout the treatment zone and a predictable area of treatment, since protons travel in a straight line and stop after a certain distance based on the initial energy imparted. Figure 5

No handling of radioactive material is required by the ophthalmologist or the radiation oncologist dealing with PBRT, in contrast to brachytherapy where handling is required. The highly collimated beam of irradiation includes a 1 mm tumour margin, a 0.5 mm margin for patient movement and a 1 mm margin for de penumbral effect. Seventy percent of the maximum radiation dose is delivered by the entrance beam as it travels through the eye before reaching the tumour. Therefore anterior complications including epiphora, lash loss and neovascular glaucoma occur more frequently with charged particles than with radiation delivered posteriorly⁷.

As of December 2002 more than 3000 patients with uveal melanoma have been treated with protons at the Massachusetts General Hospital. The 5-year actuarial local control rate is 96% for all sites within the globe, with an 80% survival rate. The probability of eye retention at 5 years was estimated to be 90% for the entire group and 97%, 93% and 78% for patients

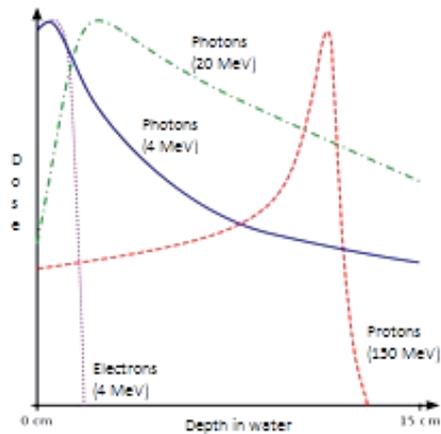


Fig. 5.

with small, intermediate and large tumours respectively. Independent risk factors for enucleation were the involvement of the ciliary body, a tumour height greater than 8 mm and the distance between the posterior tumour edge and the fovea⁸. These results compare favourably with local control rates of 89% reported with protons in Nice, France⁹, and similar results from the Paul Scherrer Institute in Villigen, Switzerland.¹⁰

Because some patients have experienced deteriorating vision after doses of 70 CGE, a randomized trial of 50 versus 70 CGE for small and intermediate-sized lesions located within 6 mm of the optic disc of the macula was conducted. Interim analysis of 188 patients, with a median follow-up of 60 months suggested no reduction in local control or survival. No significant improvement in visual outcomes or complications was observed. However visual field analysis showed a smaller mean defect in the patients randomized to 50 CGE¹¹.

Egger et al. reported long-term results of eye retention after treatment of uveal melanoma with proton beam radiotherapy¹². A total of 2645 patients (2648 eyes) were treated at the Paul Scherrer Institute in Switzerland between 1984 and 1999. The overall eye retention rates at 5, 10 and 15 years after treatment were 89, 86 and 83% respectively. Enucleation was related to large tumour size, mainly tumour height, male gender, high intraocular pressure and a large degree of retinal detachment at treatment time.

Gragoudas et al. reported that radiation maculopathy occurred in approximately 75% of tumours within 1 disc diameter of the fovea and in 40% of tumours greater than 1 disc diameter from the fovea treated with PBRT¹¹.

Wilson and Hungerford found local recurrence of 5.2% with PBRT. Metastatic death rates of 12.8% at 5 years and 20.7% at 10 years were reported following PBRT¹³.

9. Stereotactic radiosurgery

Newer techniques like stereotactic radiosurgery and gammaknife radiotherapy are being used at some centres; however, no long-term data is available on the efficacy or complication rates.

Gammaknife radiosurgery (GKR) was initially introduced to successfully treat intracranial lesions such as brain tumours, vascular abnormalities, skull base tumours and neurological

functional diseases. Gammaknife radiosurgery has been shown to be an alternative to enucleation for the treatment of large uveal melanomas.

10. Methods

10.1 Extraocular muscle sutures

After the patient receives retrobulbar anesthesia with long-acting agents (5 cm³ of 1% Ropivacaine) to obtain complete akinesia, two extraocular muscles are sutured through the conjunctiva using 3.0 black silk suture. The two muscles are chosen according to tumour location to optimise globe position during treatment.

10.2 Stereotactic lightweight aluminium frame fixation

The stereotactic frame is attached to the patient's head with four pins lodged in the outer plate of the skull. The frame provides the coordinate system for target determination by magnetic resonance imaging.

10.3 Globe immobilisation and orientation

The threads of the two sutured muscles are fixed to the stereotactic frame to immobilise and orientate the globe. The globe is oriented in order to localise the tumour as closely as possible to the centre of the stereotactic frame. This condition may reduce magnetic resonance image distortion. Correct globe immobilisation is crucial to performing precise GKR.

10.4 Neuroimaging

High-resolution magnetic resonance (2 mm slices) with gadolinium of the brain is performed, and the images are transferred to the gamma knife three-dimensional treatment planning system (Gamma Plan). The use of gadolinium increases the definition of tumour margins in the presence of subretinal fluid and retinal detachment.

Besides the advantages of being non-invasive and easier for the patient to tolerate, radiosurgery provides a single day treatment that can be completed within a few hours^{14, 15}. No other technique offers the same convenience for the patient. Although previous studies have shown GKS to be a minimally invasive, eye-saving treatment modality for uveal melanomas, secondary enucleation is still common with this procedure. Tumour volume and tumour location are thought to be major determinants of intraocular complications after GKS. Eagan et al. proposed that tumours of the ciliary body and tumors larger than 8 mm in height are more likely to require secondary enucleation after treatment. Complications of the treatment itself may also lead to enucleation.

For a series of 81 patients who underwent GKS for the treatment of uveal melanoma, Simanová et al. achieved an 84% local tumour control rate at 10 months by applying a minimum dose of 31.4 Gy¹⁶. Similarly, Modorati and colleagues achieved 91% tumour control in a group of 78 patients treated with 30–50 Gy of radiation at the tumour periphery. However, they observed high complication rates, including enucleation (10.3%), retinal detachment (33.3%) and glaucoma (18.7%). Zehetmayer et al. used one to three fractions of GKS for 62 selected uveal melanoma patients, with a mean follow-up of 28.3 months, achieving a tumour control rate of 98%. In a previous series involving GKS, doses as high as 70 Gy were applied to the immobilized the eye during the procedure; however, it appears

that the high intraocular complication rate associated with higher doses may jeopardize the conservative advantages of GKS.

11. Other treatment modalities

11.1 Trans-scleral resection

Local trans-scleral resection has largely been abandoned in favour of more successful treatment methods. In 1986, Foulds and Damato recommended local resection for tumours of 10 to 15 mm in diameter after finding that most of the failures in their series involved tumours larger than 15 mm in diameter. They reported a 19% incidence of retinal detachment as a result of the surgery, only 41% of which responded well to repair^{17, 18}. However, 81.1% of the eyes in the COMS showed local invasion of the sclera which the authors stated argued against the advisability of scleral resection as a treatment for choroidal melanoma due to the fact that potentially viable melanoma cells are likely to remain following surgery¹⁹. Proponents of transcleral resection now recommend adjuvant brachytherapy to irradiate any remaining tumor cells²⁰.

11.2 Transpupillary Thermotherapy (TTT)

TTT uses a diode laser to deliver a beam of infrared radiation through a dilated pupil into an intraocular tumour in order to induce tumour cell necrosis²¹.

The entire surface of the tumour is covered with overlapping treatment areas extending 1.5 mm past the edge of the tumour into normal tissue. The advantages of TTT include immediate necrosis with quickly evident clinical regression, precision of treatment, and ease of treatment on an outpatient basis with local anesthesia. TTT causes less choroidal damage than plaque radiotherapy. However, TTT cannot be performed if the pupil cannot be dilated to allow passage of the beam, if the tumour is so peripheral that the edges are not visible, if opacities prevent a clear view, or if there is more than 3 mm of subretinal fluid. TTT has been used successfully in select cases where plaque brachytherapy has failed. TTT has also been combined with plaque radiotherapy in a technique called sandwich therapy, as TTT is maximally effective at the apex of the tumour and brachytherapy is maximally effective at the base^{22, 23, 24}. Moreover, TTT may show benefit in the treatment of hemangiomas and small retinoblastomas in addition to uveal melanomas.

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Surgery continues to be the mainstay treatment for melanoma localized to the primary tumor and/or lymph nodes. Results from randomized controlled trials indicate that sentinel node biopsy for the treatment of cutaneous melanoma of intermediate thickness has a beneficial effect on recurrence rates, and adjuvant radiotherapy to regional lymph node fields following surgical resection reduces loco-regional recurrence in patients at high risk of relapse. Isolated limb perfusion, electrochemotherapy, and photodynamic therapy continue to be evaluated for treatment of stage IV disease. However, the greatest excitement in new treatment has been with targeted therapies for genetic mutations. In particular, the promising results of partial and complete tumor response in stage IV disease from early phase trials of the B-RAF kinase inhibitors. This book provides a contemporary insight into the therapeutic treatment options for patients with metastatic melanoma and is relevant to clinicians and researchers worldwide. In addition, an update on current clinical trials for melanoma treatment has been included, and two chapters have been reserved to discuss the treatment of oral and uveal melanoma.

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