Melanoma: Perspectives of a Vaccine Based on Peptides

24

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Contents

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The standard treatments for patients with melanoma are surgery, chemotherapy, radiation therapy, and biologic and targeted therapy. The successful surgical removal of tumors depends on the early diagnosis of the disease. Metastatic melanoma has the poorest prognosis and a number of new agents, including cytokines, monoclonal antibodies, signal transduction inhibitors, oncolytic viruses, and angiogenesis inhibitors have been tried, generally with low percentages of positive response. Vaccines have not so far been successful in the treatment of metastatic melanoma but are a reasonable area of research.

Recently, ipilimumab, the humanized anti-CTLA-4 antibody, represents a promise in patients with metastatic melanoma. In all cases the toxicity of cancer therapeutics is high. New medications and protocols are needed and are actively being investigated in many centers. Presently, we show that peptides from readily available sources may provide a basis for the development of anti-melanoma therapeutics. Some are apoptotic or affect the migration of tumor cells in vitro, but their activation of dendritic cells and antimetastatic activity in vivo, in a melanoma experimental model, is encouraging and stimulates further investigation.

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24.1 Disease Description

Malignant melanoma is the most aggressive and treatment-resistant type of skin cancer, and its incidence has increased in fair-skinned populations worldwide for decades [1].

Despite the progress in the biology and molecular genetics knowledge of melanoma initiation and development, available treatment options have not been translated into significant improvement in the survival of patients with metastatic disease, with an estimated number in 2012, of 9,180 deaths in the United States [2].

The rapidly increasing incidence of cutaneous melanoma appears to result from changes in behavioral patterns related to sun exposure, but other factors may be involved in the pathogenesis of melanoma, such as heredity and chemical carcinogenesis [3].

The most important prognostic factor for patients with melanoma is the invasion depth of the primary lesion. A roughly inverse relationship between the diameter of the lesion and survival has also been established [4]. Treatment of early stage melanoma involves complete surgical excision of the primary tumor, which is usually sufficient to cure the disease. Lymph node mapping and sentinel lymph node biopsy indicate the extent of cancer cells spreading. Once melanoma spreads to distant and inner sites, no current treatment reliably limits the aggressive course of the disease [5]. Early detection, proper diagnosis, and new therapies are needed to restrain this potentially lethal tumor malignancy.

24.2 A Brief History

Melanoma is one of the oldest known forms of malignant tumors in humans, although historical evidence for its occurrence in antiquity is scarce. In 1960s, a reported examination of nine Peruvian Inca mummies, dated approximately 2,400 years old, showed apparent signs of melanoma, such as melanotic masses on the skin and diffuse metastases to the bones [6].

The first surgeon to reportedly operate on metastatic melanoma was John Hunter in 1787. He excised a recurrent mass behind the angle of the lower jaw of a 35-year-old male and described it as containing two distinct parts: one white firm section and the other spongy and dark in appearance [7, 8]. The French physician René Laennec described melanoma as a disease during a lecture at the Faculté de Médecine de Paris in 1804 [9]. In 1840, Samuel Cooper considered individuals with advanced stage melanoma untreatable and stated that the chance of survival depends on the early surgical removal. After about two decades, the general practitioner William Norris reported that there is a familial predisposition for melanoma development [10].

Since these initial observations, studies on the biology and treatment of melanoma have intensified, and efforts have been constantly encouraged to control the disease and prolong the survival of patients.

24.3 Origin and Etiology of Cutaneous Melanoma

Cutaneous melanoma is a tumor of neuroectodermal origin that results from the proliferation and malignant transformation of pigment-producing cells, the melanocytes, which originate from neural crest progenitors and migrate to the skin and hair follicles during embryogenesis [11].

Melanocytes are located to the basal layer of the epidermis, hair bulb, eyes, ears, and meninges, and among other functions, and contribute to photoprotection and thermoregulation by melanin production [12]. Homeostasis of melanocytes is tightly regulated by interaction with epidermal keratinocytes through a complex system of paracrine growth factors, adhesion molecules, and gap junctions [13]. Mutations in critical genes linked to cell proliferation control, production of autocrine growth factors, and loss of adhesion receptors cooperate to deregulation of intracellular signaling in melanocytes. Such mutations enable cells to escape from the control exerted by keratinocytes, creating a conducive microenvironment for the uncontrolled and independent proliferation of melanocytes [14, 15].

Although the precise etiology of malignant melanoma remains obscure, evidence strongly suggests that genetic and environmental interactions play a role in melanoma development. The list of host factors important in the onset of melanoma is long and includes family history of melanoma, melanoma susceptibility genes, number and type of nevi, skin type, and pigmentation [16].

Sunlight and ultraviolet radiation exposure have been attributed the most important risk factors for melanoma. In fact, epidemiological data indicate that the growing incidence of melanoma is closely related to changes in the pattern of sun exposure. The highly genotoxic UV radiation may cause DNA damage that, if not repaired, plays a central role in the pathogenesis and progression of melanoma [17].

Epidemiological studies have established that individuals with blond or red hair and pale skin, who are subjected more easily to sunburn and freckles, more likely develop melanoma than darkly pigmented individuals. Furthermore, studies have shown that exposure to UV radiation during childhood particularly increases the risk of developing melanoma [18, 19].

Multiple melanocytic nevi, dysplastic nevi, and atypical mole syndrome (AMS) are the main clinical phenotypes indicating melanoma susceptibility [20]. Accordingly, the presence of multiple common (banal, acquired) nevi, more than five dysplastic (atypical) nevi, large congenital nevi, and lesions larger than 6 mm in diameter have been shown to precede melanoma development [21, 22].

Familial melanoma refers to a frequency of two or more first-degree relatives diagnosed with this neoplastic disease. In general, patients have the first melanoma diagnosis at early age, with thinner lesions, different distribution patterns, and a high frequency of multiple primary melanomas. The lesions in family members are histologically indistinguishable from sporadic melanomas and have similar prognosis.

To date, two melanoma susceptibility genes, *CDKN2A* and *CDK4*, have been documented. *CDKN2A* encodes the cyclin-dependent kinase inhibitor 2A (CDKN2A, $p16^{Ink4A}$) also known as multiple tumor suppressor 1 (MTS-1). P16 has an important role in regulating the cell cycle. Mutations in this gene increase the risk of a variety of cancers, chiefly melanoma. The *CDK4* gene encodes the cyclin-dependent kinase 4, a component of the protein kinase complex that is important for cell cycle G1 phase progression. Three alternatively spliced variants encoding different proteins are known, as generated by *CDKN2A* transcription. Two of them encode isoforms that function as inhibitors of CDK4. The third includes another exon 1 that contains an alternate open reading frame (ARF), which encodes a protein that stabilizes p53 by interacting with MDM2. Therefore, mutations in these genes may affect relevant signaling pathways that control cell division and may confer high risk for melanoma [23, 24].

Other genes frequently mutated in melanoma include *BRAF* on the mitogen-activated protein kinase pathway and *PTEN* on the protein kinase B/Akt pathway. Nevertheless, these mutations have been reported as results of genetic disruption and not germline predisposing alterations that may contribute to melanoma-initiating events [25, 26].

24.4 Epidemiology

The incidence of cutaneous melanoma and the number of melanoma-related deaths have increased dramatically over the past few decades, and therefore, melanoma has become in many regions a public health issue. Although malignant melanoma accounts for less than 5 % of skin cancer, it causes 80 % of deaths, mainly due to its high metastatic potential [20].

Cutaneous melanoma occurs mostly in white populations and substantially less in populations of African or Asian origin with darker pigmentation. The cancer statistics in the United States revealed that 6 in 100,000 inhabitants were diagnosed with melanoma at the beginning of the 1970s as compared to 21 cases in 100,000 inhabitants reported in the late 2000s [27], thus being the fastest growing incidence of any form of cancer. Australia and New Zealand have the world's

highest incidence and mortality rates of melanoma [18]. Within Europe, a considerable variation exists in melanoma incidence and mortality among populations, but in general, the disease has also increased in this region [28].

Although male/female melanoma incidence varies in the databases in different countries, women generally have significantly longer survival than male patients [29]. The median age for melanoma diagnosis has been 55 years [18].

24.5 Signs and Symptoms

Major signs and symptoms of melanoma are skin lesions that increase in diameter or thickness, change in color, bleeding, itching, ulcer, and tenderness [30].

The ABCD criterion for melanoma recognition was proposed in 1985 and has been adopted for appraisal of potential melanoma lesions [31]. More recently, clinical data supported the ABCDE expansion to emphasize the significance of evolving pigmented lesions in melanoma progression [32]. Clinical findings of the ABCDE acronym are *A*, for asymmetry, when one half of the lesion differs from the other half; *B*, for border irregularity, which refers to notched, uneven, or blurred edges; *C*, for color variegation, regarding the presence of shades of brown, tan, and black lesions; *D*, for diameter greater than 6 mm (although some melanomas may be smaller in size); and *E*, time evolving progression of the lesion as a cardinal feature that characterizes malignant melanoma. Survey and monitoring of lesions exhibiting these features have improved and enhanced the ability of physicians to recognize melanomas at early stages during routine skin examination, thereby substantially contributing to definitive surgical treatment.

24.6 Staging and Classification

Based on changes during melanoma progression, a microstaging system was proposed by Clark et al. [33], reflecting melanocyte proliferation during the formation of nevi, nevi dysplasia, hyperplasia, invasion, and metastasis [34, 35]. Thus, according to the model, beginning and progression of melanoma involve a series of histologic alterations that are classified into five different stages: (1) development of benign nevus, a lesion characterized by limited growth of melanocytes; (2) dysplastic nevus, characterized by discontinuous and random arrangement of melanocytes; (3) radial growth phase (RGP), characterized by intraepidermal cell proliferation; (4) vertical growth phase (VGP), characterized by melanocytes penetration through the basement membrane towards the dermis and subcutaneous tissues; and (5) metastasis of melanoma, characterized by dispersion into other areas of skin and/or organs, more commonly liver, lung, bone, and brain [36]. Breslow's depth criterion is another method to assess melanoma microstaging [37]. By use of an ocular micrometer to measure the vertical thickness of the primary tumor, it evaluates the extent of melanoma invasion with inference on disease prognosis.

Currently, both Breslow's and Clark's staging parameters have been incorporated in the American Joint Committee on Cancer (AJCC) melanoma staging system [38]. It uses the widely used TNM (*T*umor, *N*ode, *M*etastasis) parameters: *T*, for features of the primary tumor, according to Breslow's depth, cell mitoses, and ulceration; *N*, for the involvement of regional lymph nodes; and *M*, the presence of metastasis at distant sites. Additionally, serum lactate dehydrogenase (LDH) has been chosen as an important marker to evaluate the metastatic potential of the disease [39]. Cutaneous melanoma is thus classified into the following stages according to the revised AJCC guidelines [40]:

- Stage 0, characterized by the presence of abnormal melanocytes restricted to the dermis, also referred to as melanoma in situ. Defined as Tis, N0, M0.
- Stage I, characterized by tumor thickness (<1 mm), mitotic index, and ulceration status. Defined as T1a, N0, M0; T1b, N0, M0; or T2a, N0, M0.
- Stage II, also characterized by tumor thickness (>1 mm) and ulceration status. There is no evidence of lymph node involvement or

distant metastasis. Defined as T2b, N0, M0; T3a, N0, M0; T3b, N0, M0; T4a, N0, M0; or T4b, N0, M0.
- Stage III, characterized by regional lymph node involvement and micro- or macrometastasis. Defined as any T, N1, M0; any T, N2, M0; or any T, N3, M0.
- Stage IV, characterized by the presence of distant metastases and the level of serum lactate dehydrogenase (LDH). Defined as any T, any N, M1.

The melanoma staging system accurately reflects the biology of melanoma on the basis of clinical outcome, predicts behavior of this challenging neoplasm, and provides a valuable tool for clinical decision making.

Analyses of the degree of sun exposure and the associated molecular alterations in combination with histomorphological features have defined distinct patterns of genomic mutations among different groups of primary melanomas [41]. Comparative studies showed that acral (palms, soles, and subungual sites) or mucosal melanomas exhibit higher frequency of chromosomal aberrations (e.g., focal amplifications and losses) than other groups of melanoma with or without chronic sun-induced lesions [42, 43]. Furthermore, BRAF mutations have been commonly found in melanomas arising in areas intermittently exposed to the sun and rarely in melanomas on skin with chronic exposure [44]. In melanomas with V^{600} -mutated BRAF, inactivation or loss of PTEN has been detected and associated with increased Breslow thickness of lesions [45]. Classification based on molecular biomarkers may redefine diagnostic and prognostic categories, thus providing additional information on malignant melanoma treatment and management.

24.7 Diagnosis and Classical Therapy

During routine skin or physical examination, the practitioner should be alert to signs of a potential melanoma lesion according to the ABCDE criteria. Because melanoma is potentially curable when detected at an early stage, any suspicious lesions should be surgically excised and submitted to histopathological evaluation [46]. Sentinel-node biopsy is recommended as a nodal staging procedure in patients with primary melanoma, 1–4 mm in thickness [47]. Melanoma diagnosis is based on general morphological, epidermal, dermal, and cytological features, which have been closely associated with prognostic information [48].

Most primary melanomas can be treated successfully by surgical excision when detected at early stages (0, I, II) [49]. Despite decades of clinical research, prognosis for patients with advanced melanoma remains extremely poor, and available treatment options are generally not effective. In patients with stage III melanoma with involvement of regional nodes, complete lymphadenectomy is typically recommended. Following surgical resection, adjuvant therapy with interferon-alpha (IFN-α) is an option [46]. More recently, treatment with anti-CTLA-4 monoclonal antibody (mAb) ipilimumab for patients with unresectable stage III melanoma has achieved statistically significant improvement in clinical trials [50] and thus has been indicated as first- or second-line treatment [51]. Also, the use of specific BRAF mutation inhibitor, vemurafenib, in patients with BRAF V^{600} mutation-positive and unresectable melanoma has shown partial responses and might be an alternative treatment for stage III disease [52].

Among patients with stage IV metastatic melanoma, the estimated median survival time is about 8 months, and the 5-year survival rate is less than 10 % [53]. There is no consensus on the standard management of metastatic melanoma [54], but systemic therapy is the only option [55]. Dacarbazine is the most widely used single chemotherapeutic agent and in combination with immunotherapy (e.g., ipilimumab, interleukin-2 (IL-2)) may slightly improve the response rate [48]. In addition, vemurafenib recently received approval for the monotherapy of patients with BRAF $V^{600}E$ mutation as detected by a US Food and Drug Administration (FDA)-approved test [52], but clinical studies have demonstrated only a discreet improvement

in progression-free survival and overall survival [56]. Other BRAF inhibitors, such as PLX4032 (Plexxikon/Roche), RAF265 (Novartis), XL281 (Exelixis), and GSK2118436 (GSK), are in clinical trials for metastatic melanoma. Particularly with PLX4032, encouraging effects have been obtained in stage IV melanoma carrying the V^{600} BRAF mutation, with complete or partial tumor regression in the majority of patients [57]. It has been reported, however, that tumors can acquire resistance to PLX4032 treatment [58]. Recent findings have suggested that chronic BRAF inhibition mediates melanoma survival through enhanced IGF-1R (insulin-like growth factor receptor 1) signaling, a pathway implicated in regulation of cell proliferation, prevention of apoptosis, and resistance to therapy in neoplasias [59]. Altogether, these observations may suggest that targeting a single pathway may not be sufficient to eradicate melanoma.

24.8 Emerging Antitumor Peptides

Natural peptides with antitumor activity have been described from a variety of sources. Some of them are free molecules and others are internal sequences from proteins that are liberated by proteolysis or are chemically synthesized. Their antimicrobial and antitumor activities resemble ancient molecules of innate immunity that have been effective in the protection against threatening conditions, infective or others, before the emergence of antibodies and adaptive T cell immunity.

Polonelli et al. [60] identified internal sequences in an anti-idiotypic antibody that exhibited anti-infective activities, and in a collaborative study we showed that CDRs from different monoclonal antibodies showed cytotoxic activities against *Candida* and HIV but also against the highly aggressive murine melanoma B16F10 [61]. Further work confirmed that, independently of antibody specificity, internal sequences of immunoglobulins, even from the constant (Fc) region, could display anti-infective and antitumor activities, therefore being a source of bioactive peptides [62, 63]. Specifically, V_H CDR 2 (H2) from mAb C7 (C7H2), directed to a mannoprotein from *C. albicans*, and V_L CDR 1 (L1) from mAb HuA (HuAL1), directed to human blood group A, tested as C-amidated synthetic peptides, led melanoma cells to apoptosis in vitro. Further, they were protective in a metastatic syngeneic model in vivo using intravenously challenged C57BL6 mice. C7H2 peptide (YISCYNGATSYNQKFK) exerted its apoptotic activity not only in B16F10 murine melanoma cells but also in several human tumor cell lines at similar EC50 concentration. Recently, Arruda et al. [64] showed that C7H2 bound to and caused polymerization of G-actin while acting on F-actin to stabilize it and, thus, altering actin dynamics which led to apoptosis following an intense production of superoxide anions. A typical caspase-dependent apoptosis ensued, documented by a number of cellular alterations and organelle disruptions. Similar effects were found with HuAL1 (RASQSVSSYLA), which apparently binds to a different receptor and causes tumor cell death with characteristics of necrosis (Arruda D.C., 2013 unpublished data).

Monoclonal antibodies to B16F10 murine melanoma (A4 and A4M) also provided CDR peptides with antitumor activity [63]. Interestingly, the V_H CDR 3 (H3)'s of both mAbs acted as microantibodies competing with the mAbs for binding to melanoma cells. A4H3 peptide in a linear or cyclic-extended form was as cytotoxic as mAb A4, targeting protocadherin β-13 (highly similar to human protocadherin β-6) on murine melanoma cells. The linear A4 H3 peptide (IRDGHYGSTSHWYF) was able to inhibit melanoma cells, at EC50 0.06 mM. Similarly with A4, it induced DNA degradation in B16F10-Nex2 cells and was also active in vivo against metastatic melanoma. CDRs L1 (RASGNIHNYLA) and L2 (NVKTLA) from mAb A4M inhibited growth of B16F10-Nex2 cells and induced DNA degradation in both melanoma and HL-60 cells, counteracted by Bcr-Abl, Bcl-2, and Bcl-X_L hyperexpression in the latter. The apoptotic effect of both L1 and L2 was accompanied by the anti-angiogenic activity of both peptides using HUVEC as target cells [63].

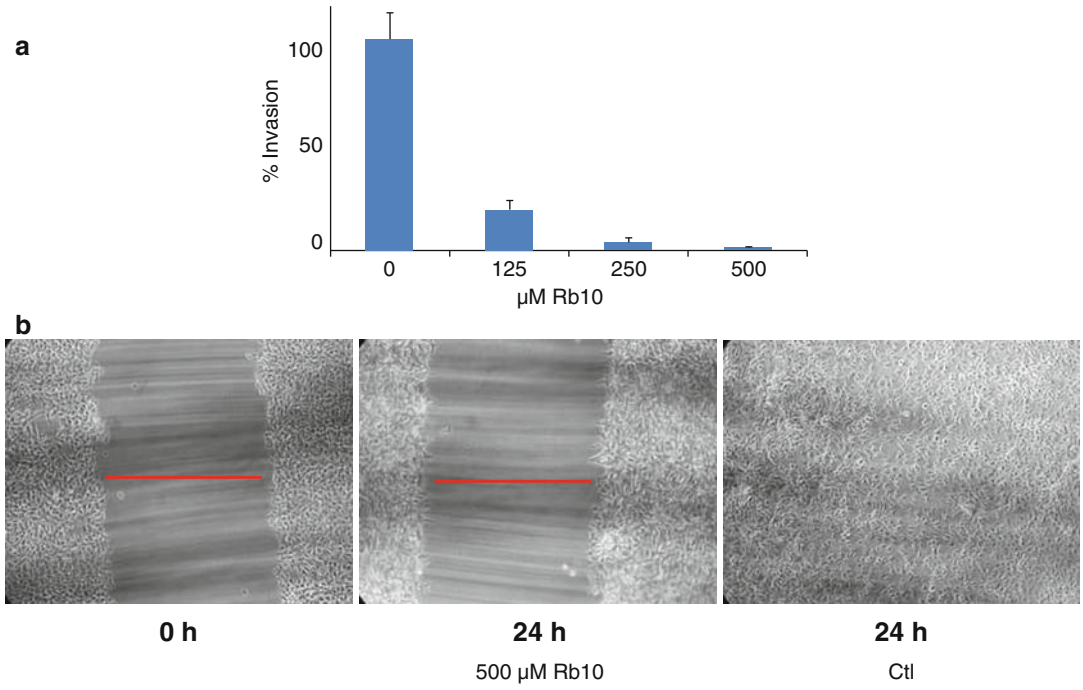

Fig. 24.1 Inhibition of melanoma cell invasion (Matrigel) and migration by Rb10 peptide (H3). B16F10-Nex2 melanoma cell invasion of Matrigel was measured in Transwell chambers, with 2×10^5 tumor cells suspended in serum-free medium added on the top chamber and 10 % FBS in the bottom chamber serving as cell attractant. Migrating cells were fixed, stained with Giemsa, and counted. (**a**) Inhibition of cell invasion by Rb10 at the indicated concentrations after 24 h. Values are from triplicate determinations. (**b**) Inhibition of B16F10-Nex2 melanoma cell migration (3×10^5 cells allowed to reach 70–80 % confluence in 12-well plates) by Rb10 using the scratch wound-healing assay. Complete inhibition was observed after 24-h incubation compared to the untreated control

The CDR H3 peptide from mAb REB 200 (humanized mAb MX35 directed to sodium-dependent phosphate transport protein 2b, NaPi2b), named Rb10, and its cyclic-extended derivative Rb9 are not apoptotic but induced a remarkable effect on tumor cells. They caused hyperadherence of cultured cells, thus inhibiting both migration and invasion through Matrigel (Fig. 24.1). Rb10 was antimetastatic in vivo using the murine melanoma model and apparently acted on tumor cells through HSP90 binding and inhibition of cell motility (PTC/1B2011/03053).

Another effect attributed to CDR (H3) of an anti-blood group A murine mAb (Ac1001) was the immunomodulation of macrophages [65]. The H3 peptide stimulated cytokine production, activated PI3K-Akt, and enhanced the expression of TLR-4. The different properties unraveled for the CDR peptides strengthened their potential use as medicinal drugs [66]. A list of apoptotic/necrotic peptides against several tumor cell lines is given on Table 24.1.

24.9 In Vivo Activity of Peptides and the Perspective of Antitumor Vaccines

The discovery of the apoptotic effect of immunoglobulin CDRs in vitro, targeting several tumor cell lines rather than nontumorigenic cells, led us to ask whether their protective antitumor activity in vivo could involve the same mechanism. Free peptides, even with the carboxy-terminal protected by amidation, are generally not very stable in the circulation being substrates of plasma peptidases and, depending on the size, cleared by renal filtration. Nevertheless, peptides of 16 (C7H2) and of 11

Table 24.1 CDR peptides from mAbs of various specificities which display apoptotic/necrotic antitumor activities

mAb	Immunogen	Ig	CDR	References
A4	Melanoma B16F10-Nex4	IgG	A4 CDR H3 (mic.mAb)	[64]
A4M	Melanoma B16F10-Nex4	IgM	A4M CDR L1 and L2	[64]
C7	Mannoprotein of *Candida*	IgM	C7 CDR H2	[62, 65]
Pc42	Hepatitis B/*P. falciparum* hybrid	IgM	pc42 CDR H2	[62]
HuA	Human blood group A (Fuc_2)	IgM	HuA CDR L1	[62]
AC1001	Mouse anti-blood group A (Fuc_2)	IgM	Ac1001 CDR H3	[66]
C36	Vaccinia	IgG	C36 CDR L1	–

The H3 may act as a micro (mic) antibody

(HuAL1) amino acids administered by intraperitoneal injections and in alternate days conferred significant protection against B16F10 tumor cells in a metastatic model [61].

An immune response to both peptides was suggested, and in the case of C7H2, the lack of activity in vivo of peptides with alanine substitutions at the N-terminal (Y1A and C4A) was compatible with specific amino acid recognition [64]. The apoptotic effect of C7H2 was associated to β-actin binding, but the C-terminal sequence rather than the N-terminal of C7H2 was shown to be involved in this reactivity.

The importance of the immune system was shown by the ineffectiveness of C7H2 to protect against melanoma in a metastatic model in NOD/Scid/IL-2rγ^{null} mice, implying that cells of the immune system were involved.

To explore the participation of the immune response, we examined the protective activity of dendritic cells stimulated or not by the in vitro-apoptotic CDRs. The same metastatic model of B16F10 melanoma cells was used. Syngeneic dendritic cells from C57Bl6 mice when primed with melanoma antigens were partially protective, but these cells pre-stimulated with the CDRs displayed a very significant antitumor effect (Fig. 24.2). It seems then that the antitumor effect in vivo does not depend on the direct targeting of tumor cells by the peptides but that dendritic cells may amplify the antitumor effect before the natural clearance of the CDRs. We hypothesize that CDR peptides, before degradation, are efficiently taken up by dendritic cells and that activated DCs most efficiently present tumor antigens for a competent T lymphocyte CTL response. A more extensive investigation has to follow these observations including other apoptotic/necrotic peptides to determine the effector cells and other characteristics of the protective immune response.

It is doubtful whether the protective in vivo effects of A4H3, a microantibody sequence from apoptotic mAb A4 and of Rb10 (and Rb9), CDRs that inhibit tumor metastasis (Fig. 24.3), are also dependent on DCs, but this has not yet been determined.

24.10 Antitumor Peptides Derived from Signaling Proteins

Apart from CDRs, which may concentrate their bioactivity owing to the high diversity and variability of their amino acid sequences, other peptides derived from signaling proteins have emerged as potential anticancer agents. Some of them are active in vivo and not in vitro like those of SOCS1 (Scutti J., 2013 unpublished results), and some others have several targets and complex mechanism of action still under investigation. Presently, we mention peptide pTj derived from the C-terminal zinc finger domain of WT1 [67]. WT1 is a transcription factor associated with melanoma oncogenesis and proliferation. The pTj peptide showed antiproliferative activity linked to irreversible G2/M cell cycle arrest and induction of senescence. In vivo, pTj

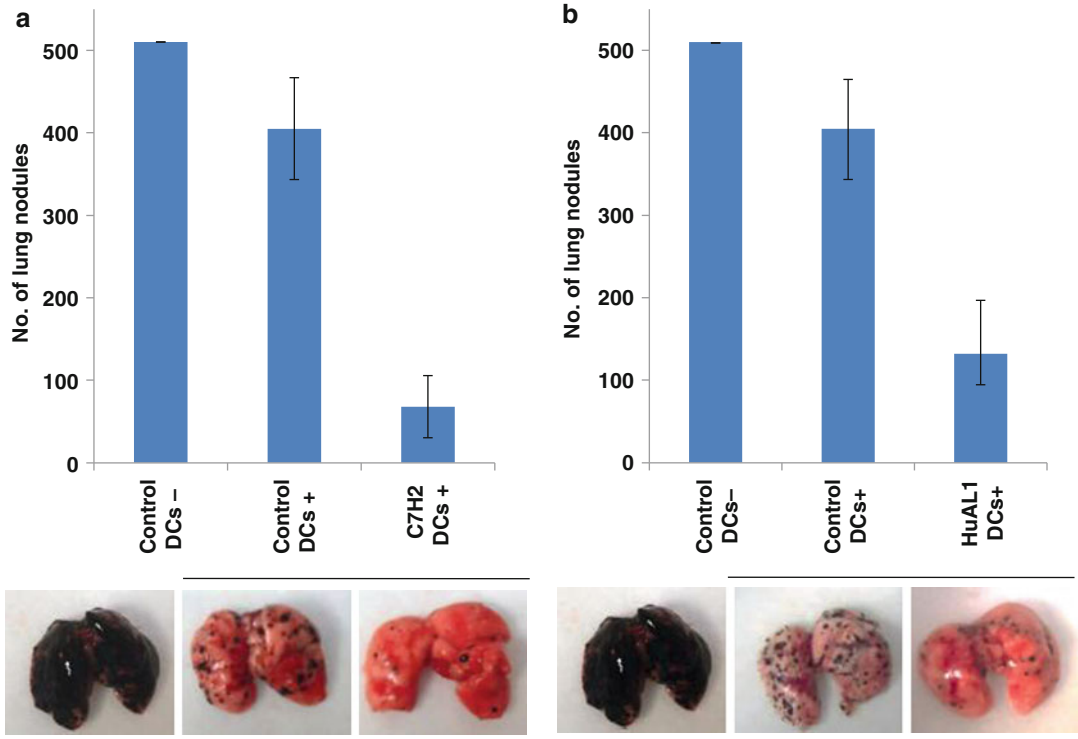

Fig. 24.2 Protective peptide-activated dendritic-cell vaccine against metastatic melanoma. Bone marrow dendritic cells (*DCs*) from syngeneic C57Bl/6 mice were primed with melanoma antigens (lysate from 5×10^5 B16F10-Nex2 cells incubated with 3×10^5 DCs for 1 h). Primed DCs were incubated with 20 μg/ml of either C7H2 or HuAL1 for 3 h. Peptide-stimulated DCs were injected subcutaneously in mice on days 1 and 4. Animals were challenged on day 11 with 5×10^5 melanoma cells injected endovenously. (**a**) protective effect of the C7H2-DCs primed (+) with melanoma antigens. Control primed (+) DCs but nonactivated with the peptide were poorly effective compared to control unprimed, nonactivated DCs. (**b**) the same as in (a), but with HuAL1 peptide-activated DCs. Images of the lungs with melanoma nodules as shown

showed a significant antitumor effect reducing the number of lung nodules in the B16F10 syngeneic model of metastatic melanoma and prolonging survival of nude mice challenged subcutaneously with human melanoma A2058 (Fig. 24.4).

Natural peptides derived from immunoglobulins and from domains of signaling proteins may represent a phylogenetic counterpart to peptide sequences from early innate immunity molecules that played an important role in the defense and regulatory mechanisms, with several reminiscent molecules still exerting important functions in our days. When tested as isolated peptides, they can act by themselves or through the activation of effector cells such as those of the immune system.

24.11 Antigen Characterization

Traditionally immune peptides are readily characterized after being mapped in antigenic proteins. In the case of human melanoma, MHC-restricted peptides from TRP2, NY-ESO-1, Melan A/MART-I, tyrosinase, and gp100/Pmel17 have been used as immunogens. Due to the heterogeneity of antigen expression, several groups tried whole melanoma cells expressing cytokines by gene transduction. Most promising is the use of genetically engineered lymphocytes reactive with NY-ESO-1 [68]. The NY-ESO-1 cancer/testis antigen is expressed in 80 % of patients with synovial cell sarcoma and approximately 25 % of patients with melanoma. Blockade of CTLA-4 increases CD8 (+) T cells

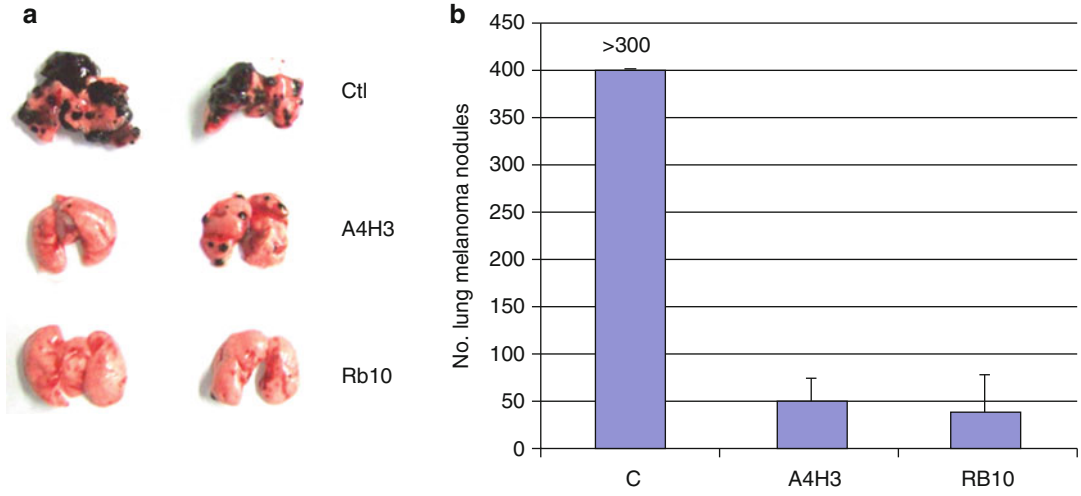

Fig. 24.3 Antimetastatic effect of peptides A4H3 and Rb10. Syngeneic mice (C57Bl/6) were challenged with 2×10^5 B16F10-Nex2 melanoma cells in 100 **µ**l, endovenously. Peptides were administered in six doses of 250 **µ**g/mouse/day intraperitoneally, starting on day 1 after challenge. Mice (5 per group) were sacrificed 22 days post-challenge and 10 days without treatment with the peptide. (**a**) lungs from untreated animals and from peptide-treated mice; (**b**) melanoma nodules in the lungs of control (untreated) and peptide-treated animals

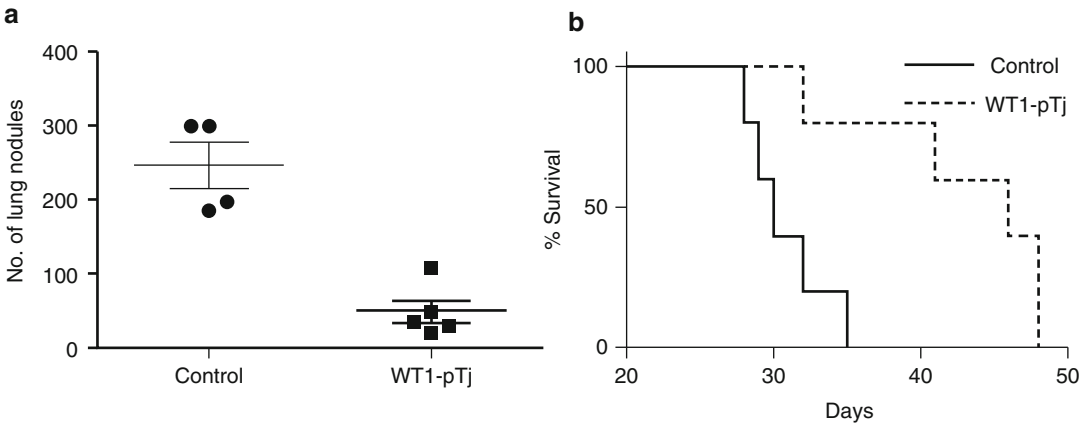

Fig. 24.4 Protective effect of WT1 pTj in vivo. (**a**) Lung colonization of B16F10-Nex2 cells in a syngeneic system was used to test the protective activity of WT1-derived peptide. Animals received 5 intraperitoneal doses of 300 **µ**g of WT1 pTj or vehicle (PBS) on consecutive days. The number of lung metastatic nodules was significantly reduced by 81 % after WT1 pTj treatment. (**b**) The survival curve of nude mice bearing human melanoma and treated with the WT1-derived peptide. Animals received 300 **µ**g of WT1 pTj or vehicle (PBS) on consecutive days. Peritumor therapy started the day after inoculation of melanoma cells and was extended for 5 days. Mean survival times were 31 ± 2.7 days for untreated tumor control group and 43 ± 6.7 for WT1 pTj-treated group

to a broader array of preimmunized melanoma antigens [69].

In the case of the peptides of the present review, they do not cross-react with tumor antigens to elicit a specific antitumor response. They act directly on cancer cells binding to different receptors or indirectly, activating cells of the immune system.

As said above, CDRs C7H2 (pc42H2) and A4H3 recognize β-actin and protocadherin β-13 (human β-16), respectively. Rb10M3, a derivative of Rb10 with dA at the second position,

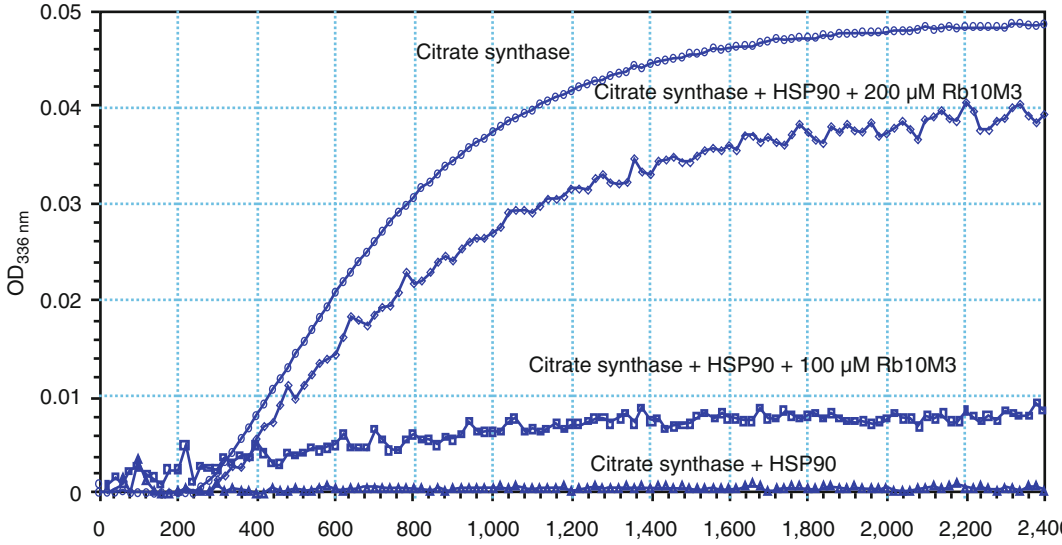

Fig. 24.5 Dose-dependent inhibition by Rb10M3 of HSP90 chaperone function using a citrate synthase aggregation assay. Rb10M3 is a derivative of Rb10 with dA (A2dA) to increase stability. Citrate synthase (0.115 **μ**M), in 40 mM HEPES, pH 7.5, was incubated at 43 °C for denaturation and aggregation. Aggregation was measured at OD 336 nm

reacts with HSP90, inhibiting its protein anti-aggregating activity (Fig. 24.5). Interference in the chaperone property of HSP90, which is highly expressed in tumor cells, affects a number of signaling pathways in the cell. In line with Rb10 inhibition of tumor cell metastasis, it was shown that actin dynamics-mediated cellular protrusion responsible for cell migration and invasion of matrix was associated with HSP90 inhibition [70]. HuAL1 binds to histone-3. WT1 pTj enhanced p53 activity and competed with WT1 protein for binding to p53 [67]. WT1 pTj may prevent important interactions between WT1 protein and its partners via a zinc finger motif. The peptide is, therefore, a promising agent against WT1-expressing malignancies (e.g., MCF-7, but not SK-BR-3 breast cancer or normal human fibroblasts, and also HL-60 acute myeloid leukemia).

24.12 Animal Model

C57Bl/6 mice and syngeneic murine melanoma B16F10 (subline Nex 2, developed at the Experimental Oncology Unit of Federal University of São Paulo, UNONEX-UNIFESP) are generally used for in vivo experiments. Melanoma cells are injected subcutaneously (4×10^4 to 10^5 cells) and tumor growth in mm^3 is measured applying the formula: $V=(d)^2\times D\times 0.52$ (wherein V is the tumor volume, D is the longest diameter, and d is the shortest diameter). For the metastatic model (lung colonization), 10^5 to 5×10^5 cells of B16F10-Nex2 melanoma are injected endovenously, and after 15–20 days the animals are sacrificed and the black nodules counted in the lungs. Peptides used in the protection experiments are injected intraperitoneally at 150–350 μg/mouse/day. Depending on the protocol, peptides are administered on alternate days, and treatment may be interrupted after 11–15 days for plotting the survival curve. For certain experiments with B16F10 and with human tumor cells, immunological-deficient animals are used. Nude$^{-/-}$, RAG 1/2$^{-/-}$, and NOD/Scid/IL-2rγ^{null} mice have been used with the tumor cells injected subcutaneously (A2058, SKMel28 human melanoma cells) or endovenously (B16F10-Nex2 murine melanoma cells). Peritumor injections were used for the protection experiments with WT1 pTj peptide.

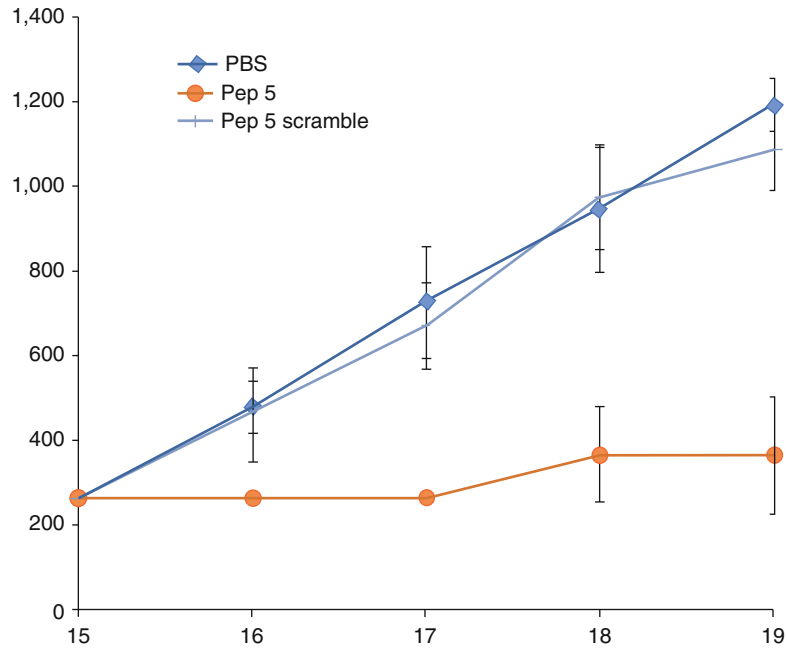

Fig. 24.6 SOCS-1-derived peptide (P5) arrests subcutaneous tumor development. Tumors were produced by injecting 5×10^4 B16F10-Nex2 cells into the right flanks of C57BL/6 mice ($n = 5$ per group). After 15 days (tumor size of 200 mm^3), the animals were treated i.p. for five consecutive days with 300 **µ**g peptide/mouse (*$p < 0.01$)

Prophylactic and therapeutic protocols were used in these studies. With SOCS-1 peptides, a therapeutic protocol started after the subcutaneously grafted tumor had reached 200 mm^3 (Fig. 24.6). In this model, 5×10^4 B16F10-Nex2 cells were injected into the right flanks of C57BL/6 mice ($n = 5$), and after 15 days the animals were treated intraperitoneally for 5 days with 300 µg peptide/mouse. As shown, tumor growth was arrested with SOCS-1 P5 treatment. The scramble peptide was used as a negative control.

24.13 Feasibility and Preclinical Development

Peptides listed on Table 24.1 and those derived from SOCS-1 and WT1 are readily obtainable and can be prepared in high degree of purity and reproducibility. Derivatives of these peptides will depend on the complexity of chemical synthesis. Conjugates with nanoparticles or macromolecules are more easily obtainable if validated in terms of antitumor activity.

Several peptides herein described depend on immune cells for their in vivo protective effects, and presently, we show two of them that acted via activation of dendritic cells (DCs). Activation in vivo of DCs by peptides and the activity of these cells as therapeutic agents is a possibility that has many precedents in DC-based vaccine trials using different propagation methodologies, antigenic load, and cell administration [71]. In the 1990s tumor peptides or whole-cell antigens have been used for ex vivo pulsing of DCs [72, 73]. In contrast, the peptides reviewed here are unrelated with the tumor cells and apparently act directly on DCs, rendering increased effectiveness to these APCs. They seem to activate DCs in vivo, which is an essential condition for triggering a protective immune response. In metastatic melanoma, vaccination with DCs loaded with melanoma antigen resulted in 9.5 % tumor regression compared to a maximum of 4.6 % using tumor cells, 2.7 % with immune peptide vaccines, and 1.9 % with viral vectors [74]. In our experiments in vivo simple injections of the peptides were sufficient to protect mice from metastatic melanoma, implying that tumor antigen presentation by activated DCs had been effective. As shown, protection was reproduced by peptide-treated tumor-antigen primed DCs ex vivo (Fig. 24.2). Further, it has been shown that intravenous or subcutaneous immunization with tumor

peptide-pulsed DCs in a mouse model of melanoma induced memory T cells that enabled control of metastasis in the lungs [75].

Comparison of in vivo DC-targeting and ex vivo antigen-loaded DCs has been made by Bonifaz et al. (2004) using B16 melanoma. Mice were challenged with B16 expressing OVA and then vaccinated with anti-DEC-205-OVA in the presence of anti-CD40. This was compared with ex vivo OVA-loaded mature DCs. The latter immunization did not inhibit tumor growth. Vaccination with anti-DEC-205-OVA was effective in controlling tumor growth. In the case of the antitumor peptides described in the present review, no targeting receptor has been identified so far, but there is direct evidence that DCs are activated by them. On the other hand, except for peptides derived from SOCS-1, the CDR-derived peptides and that derived from WT1 seem to recognize different ligands although, apparently, exerting similar stimulating effects.

If DCs are indeed those cells primarily targeted by the antitumor peptides, preclinical data in the mouse have to be translated into successful outcomes for patients. Identification of conserved targets in murine and human cells is an important step in these studies to understand the relation between immune modulation and vaccination.

24.14 Strengths and Weakness

Short-size peptides are usually poorly stable in vivo owing to proteolytic degradation and renal filtration. Yet, the antitumor peptides reviewed here, inoculated intraperitoneally in alternate days, are protective against metastatic melanoma. Such protection is not observed in immune-deficient, mice implying that cells of the immune system are necessary. Direct evidence was obtained for the activation of bone marrow dendritic cells which when primed with melanoma antigens ex vivo effectively protected against metastatic B16F10 melanoma. We hypothesize that injected peptides are rapidly taken up by dendritic cells, before being cleared from the blood circulation. Peptide-activated DCs would be quite efficient in the task of presenting tumor antigens even without targeting these cells in vivo with antibodies as discussed by Caminschi et al. [76]. Further, the types of DCs activated by these peptides are competent to induce T cells able to control tumor growth. Recently, Scutti in our laboratory (Scutti, JAB, 2013 unpublished results) showed that injection of a SOCS-1 peptide in mice challenged with B16F10 melanoma produced spleen T-CD8+ cells that were protective against metastatic melanoma growth in $RAG^{-/-}$ mice.

The main weakness of the present study is its early stage of development. Numerous questions have still to be answered mainly related to the mechanism of action of the peptides, their biodistribution, and the types of immune cells activated for antitumor effects. The peptide receptors and how the experiments in the mouse melanoma model can be translated into clinically effective procedures are other important concerns that will demand intense investigation. Nevertheless, the simple existence of these peptides, their easy production in purified state, and their anticancer effects warrant the present investigation.

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