Angiogenesis and Metastasis Inhibitors for the Treatment of Malignant Melanoma

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Abstract: Malignant melanoma is one of the most highly invasive and metastatic tumors. Melanoma is an increasingly common malignancy as well, and its mortality rates have been rapidly increasing above those of any other cancer in recent years. Surgical resection and systemic chemotherapy are the main therapeutic strategies for the treatment of malignant melanoma. However, these approaches are insufficiently effective and may be associated with significant adverse effects. Angiogenesis, a process by which new vascular networks are formed from pre-existing capillaries, is required for tumors to grow, invade and metastasize. Tumor vessels are genetically stable, and less likely to accumulate mutations that allow them to develop drug resistance in a rapid manner. Therefore, targeting vasculatures that support tumor growth, rather than cancer cells, is considered the most promising approach to malignant melanoma therapy. Now, novel anti-angiogenic agents with tolerable side effects is actually desired for the treatment of patients with malignant melanoma. In this paper, we review the current understanding of anti-angiogenic therapy for malignant melanoma, especially focusing on pigment epithelium-derived factor (PEDF), which was recently identified as the most potent endogenous inhibitor of angiogenesis in the mammalian eye. We also discuss here the involvement of a receptor for advanced glycation end products (RAGE) in angiogenesis, melanoma growth and metastasis, and the therapeutic implications of the blockers of RAGE in this devastating disorder.

1. INTRODUCTION

Malignant melanoma is an increasingly common public health problem in many countries. It results from the malignant transformation of melanocytes (Fig. **1**). Since the mid 1960s, melanoma incidence has risen by 3–8% per year in most people of European origin, with the greatest increases in elderly men [1]. Despite this increase, and an overall rise in mortality due to melanoma, the survival rate has improved substantially. Roughly 60% of those diagnosed with melanoma in the 1960s died of the disease, compared with just 11% more recently, an improvement attributed mainly to early detection [2]. Main treatments for melanoma are total excision of the tumor and early evaluation of metastasis including sentinel node biopsy. However there have been no effective systemic therapies once melanoma starts to grow rapidly and metastasize, nevertheless many regimens for multiple chemotherapies, radiotherapy and immunotherapy have been tried. Melanoma patients with visceral involvement have a median survival of only 4-6 months [2]. Thus controlling the tumor growth and metastasis is one important clue to improve the extremely poor prognosis of patients with progressed melanoma.

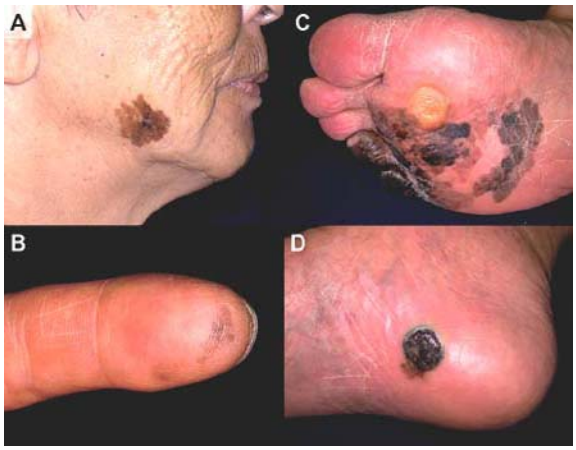

Fig. (1). Clinical images of malignant melanoma. In early stage, melanoma is an enlarging brown macule with color variegation on the face (A, also termed as lentigo maligna) and extremities (B). In advanced stage, vertical proliferation causes elevation and ulceration (C), or macules spread to cover large part of foot (D).

A major microenvironmental event in tumor growth and expansion is the 'angiogenic switch', an alteration in the balance of pro-angiogenic and anti-angiogenic molecules that leads to tumor neovascularization (Fig. **2**) [3]. Angiogenesis, a process by which new vascular networks are formed from pre-existing capillaries, is required for tumors to grow, invade and metastasize [4]. Tumors are unable to grow beyond a volume of 1-2 mm^3 without establishing a vascular supply because active cells must remain within 100-200 μm of a blood vessel to survive [4]. Tumor vessels are genetically quite stable, and less likely to accumulate mutations that allow them to develop drug resistance in a rapid manner [5]. Therefore, targeting vasculature that supports

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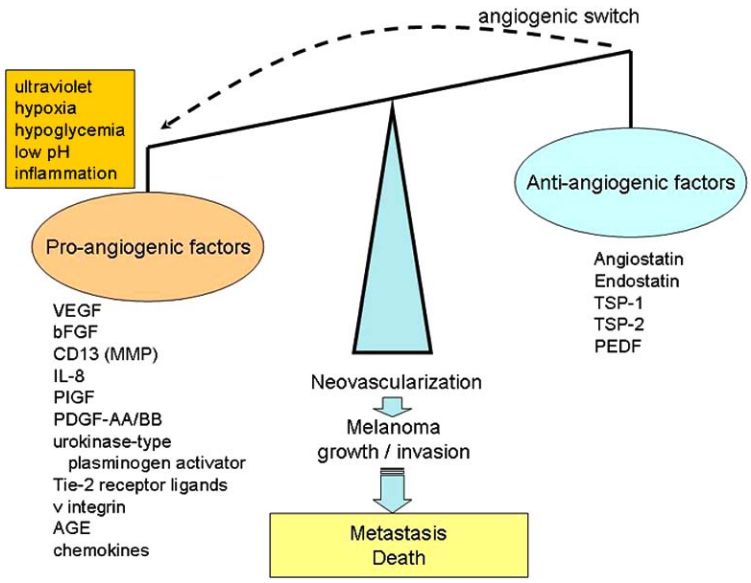

Fig. (2). A schematic outline of angiogenic switch resulting in tumor angiogenesis. There are various associated factors promoting or inhibiting angiogenesis. Both factors are greatly affected by environments, such as ultraviolet, hypoxia and inflammation. Imbalance of angiogenic switch and decline into pro-angiogenic factors causes Neovascularization, resulting in melanoma invasion and metastasis. (AGE; bFGF; basic fibroblast growth factor, IL; interleukin, MMP; matrix metalloproteinase, PIGF; placental growth factor, PDGF; platelet-derived growth factor, PEDF; pigment epithelium-derived factor, TSP; thrombospondin, VEGF; vascular endothelial growth factor).

tumor growth, rather than cancer cells themselves, is considered the most promising approach to cancer therapy.

Many tumors, including malignant melanoma not only overexpress multiple angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and interleukin (IL)-8, but also underexpress angiogenic inhibitors such as thrombospondin-1, thus favoring angiogenesis [6,7]. Among them, VEGF is the best characterized angiogenic factor involved in melanoma tumor expansion [8]; hypoxia, hypoglycemia, low intra-tumor pH, or inflammation stimulates VEGF expression in tumors. The angiogenic tumor phenotype with enhanced VEGF expression is correlated with a poor prognosis and low patient survival rates. VEGF acts on endothelial cells by binding to the membrane receptors, KDR and FLT-1. These receptors are basically expressed by endothelial cells and not by the tumor cells secreting the growth factors. Large numbers of anti-angiogenic agents with various mode of action are currently being tested and are currently under consideration for several clinical trials.

Metastasis is the transfer of malignant tumor cells from one organ to a distant organ [9]. It is the most common cause of death in cancer patients. Different molecular mechanisms enable tumor cells to infiltrate the surrounding tissue, invade into and out of blood/lymphatic vessels and leave the blood stream at a different site. Tumor cell interactions with extracellular matrix (ECM) components and epithelial-mesenchymal transition are important factors in invasion and metastasis. Gene expression profiles of metastatic melanoma cells in various organs are variable and not every tumor cell has the capacity to metastasize to a particular organ if at all. The microenvironment in the target organ can thus influence the formation of metastases.

2. ANGIOGENSIS IN MELANOMA

New blood vessel formation is an important and prominent feature of human cutaneous melanomas, indicating that these tumors have angiogenic activity [10,11]. A rise in the mean vascular density was correlated with melanoma progression in subsequent histochemical studies of cutaneous melanomas [12,13], and a number of retrospective histological studies have reported an inverse correlation between tumor microvessel density and disease-free/overall survival of melanoma patients [14,15]. A prospective study of cutaneous melanoma patients reported that tumor vascularity, as determined by analysis of routine histologic staining, was the most important determinant of overall patient survival [16].

Multiple angiogenic factors are produced by primary cutaneous melanoma cells. VEGF is an endothelial cell-specific growth factor and the principal regulator of angiogenesis under normal and pathological conditions in most organs [17], including the skin [18]. Using immunohistochemical analyses, the transition from a horizontal to a vertical growth phase in melanoma was found to be associated with increased VEGF protein expression and accumulation in the tumor stroma [12,13]. Other studies demonstrated that VEGF was only expressed in 32% of primary melanomas, with increased expression levels in metastases [15,19].

However, these studies demonstrated that VEGF expression is not as prominent in melanomas as in most epithelial cancers [20] and, therefore, might not represent the most important angiogenic activity in these tumors. The expression of functional VEGF receptors on human melanoma cells suggests the intriguing possibility that VEGF might act as an autocrine factor and exert growth-promoting effects on the tumor cells themselves [21]. Furthermore VEGF from tumor cells can change into its isoforms by different splicing, resulting in increasing angiogenic activity in early hypoxic microenvironment [22]. However, angiogenesis is not seen to occur by VEGF independently; a recent experiment reports that VEGF-induced angiogenesis depends on the existence of matrix metalloproteinase (MMP) [23].

Expression of basic fibroblast growth factor (bFGF) gene, a potent angiogenic factor, has been detected in metastatic and primary invasive melanomas, whereas melanocytes in benign nevi failed to express this factor [24]. Low bFGF serum concentrations at the beginning of high-dose interferon (IFN)-2b therapy were associated with patient recurrence-free survival [25].

CD13/Aminopeptidase N is a membrane-bound, zinc-dependent metalloproteinase that regulates the N-terminal modification of various proteins, resulting in tumor invasion by angiogenesis and degrading ECM [26-28]. CD13 is expressed on endothelial cells within human tumors, which is activated by various angiogenic conditions such as hypoxia through Ras signaling pathways [29]. Recently bFGF can induce overexpression of CD13 in human melanoma cell *in vitro* [30], suggesting an association between CD13 and melanoma angiogenesis. Other aminopeptidases such as type 2 methionine aminopeptidase, and adipocyte-derived leucine aminopeptidase/puromycin- insensitive leucyl-specific aminopeptidase are also involved in angiogenesis [31].

Several other angiogenic factors have been implicated in the pathology of human melanomas. IL-8, in particular, was absent from normal epidermis and benign melanocytic lesions but was expressed at high levels in the majority of cutaneous melanomas [32]. Moreover, IL-8 serum levels were found to be elevated, compared to healthy controls, and were correlated with an advanced disease stage and poor overall survival [33]. Immunohistochemical analysis revealed increased expression levels of the angiogenic factors: placental growth factor (PlGF; [21]), platelet-derived growth factor (PDGF)-AA and PDGF-BB [34] in human melanoma tissue samples.

Down-regulation of endogenous angiogenic inhibitors was observed in several epithelial cancers, and was proposed to enhance tumor progression. However, in contrast, to the large number of studies on angiogenic molecules, little is known about the expression of endogenous angiogenic inhibitors by melanoma cells. An inverse correlation between mutations in the tumor suppressor p53 and the expression of the angiogenesis inhibitor thrombospondin (TSP)-1 was found in a study of 99 melanoma samples. Researchers detected a significantly higher incidence of p53 mutations in metastatic tumors, suggesting that acquisition of these mutations, coupled with decreased TSP-1 expression, might promote the metastatic phenotype in malignant melanoma [35].

2.1. Therapeutic Targetting of Angiogensis in Melanoma

2.1.1. Angiogenesis in Experimental Melanoma Models

Human melanoma cells synthesize a plethora of angiogenic factors *in vitro*, including VEGF, bFGF, IL-8, PDGF, and PlGF. The specific biological function(s) of several of these factors has been evaluated in both *in vitro* angiogenesis and in xenograft models. VEGF was not expressed in normal melanocytes but upregulated in malignant melanoma cells [36]. In a series of melanoma cell lines, *in vitro* VEGF expression was correlated with the degree of tumor angiogenesis and the metastatic potential of *in vivo* tumor xenografts [37]. Overexpression of VEGF in melanoma cell lines promoted tumor growth, angiogenesis, and metastasis *in vivo* [38].

Basic FGF is expressed by most melanoma cells, but not by normal melanocytes, whereas both types of cells express high-affinity receptors for bFGF [39]. Although overexpression of bFGF conferred the capacity for anchorage-independent growth to human and murine melanocytes *in vitro*, bFGF-transfected melanocytes did not form persistent malignant tumors *in vivo* [32]. This indicates that autocrine bFGF stimulation provides a growth advantage but is not sufficient for the induction of a transformed phenotype. Downregulation of VEGF expression slowed tumor growth, whereas transfection of a bFGF antisense construct completely inhibited tumor formation, indicating an important autocrine function of bFGF in human melanoma development [40].

CD13 is also expressed strongly by melanoma cells. Inhibitors of CD13, such as bestatin and amastatin can suppress invasion of melanoma *in vitro* [26]. CD13 is also implied as a homing protein to tumor tissues with active angiogenesis. Tumor necrosis factor (TNF) alpha, a well-known anticancer agent, can work with 30 times lower doses against murine melanoma model when TNF alpha is fused with CNGRC peptide, a CD13 ligand [41].

IL-8 and PDGF have also been implicated in the promotion of experimental melanoma growth, angiogenesis, and metastasis [6]. IL-8 is produced by melanocytes and melanoma cells. It stimulates cell migration, proliferation, and metastases in an autocrine fashion, as shown in IL-8-transfected non-metastatic melanoma cells [42]. Moreover, in human melanoma xenografts, the incidence of spontaneous metastasis was associated with increased IL-8 expression, and treatment with IL-8 neutralizing antibodies significantly decreased angiogenesis and the formation of metastases [43]. In human melanoma cells that do not express PDGF, induction of tumor-associated blood vessels and formation of a dense connective tissue stroma were observed after cells were transfected with a PDGF expression vector [44].

2.1.2. Therapeutic Targeting of Angiogenesis in Melanoma Patients

More than three decades ago, Folkman proposed that inhibition of tumor angiogenesis might represent a new strategy for treating human cancers [45]. Increasing experimental evidence, obtained predominantly in tumor xenotransplan-

tion models, suggests that in addition to epithelial cancers, malignant melanoma growth and progression might also be inhibited by the blockade of blood vessel growth. Furthermore, conventional chemotherapies or immunochemotherapies can influence various angiogenic factors in melanoma patients [46], which implies that angiogenesis is a promising target for therapeutic strategies both directly and indirectly. Several reagents have been developed that block VEGF activity, including neutralizing antibodies against VEGF and small molecules or antibodies that prevent VEGF from binding to, or signaling through its receptors on the vascular endothelium cells. These have been demonstrated to have antitumor and anti-angiogenic activity in melanoma models [47]. Moreover, studies designed to block bFGF activity have been shown to inhibit tumor angiogenesis, melanoma growth, and metastasis [48].

Other anti-angiogenic strategies that have shown antitumor efficacy in preclinical melanoma models [10] include targeted inhibition of the urokinase-type plasminogen activator [49], Tie-2 receptor ligands [50], the v integrin receptor [51], and MMPs [52]. Moreover, overexpression or systemic application of the endogenous angiogenesis inhibitors angiostatin [53], endostatin [54,55], TSP-1 [56], TSP-2 [57] and pigment epithelium-derived factor (PEDF) [58] have been shown to slow tumor growth in melanoma xenograft models. In this review we focus especially on these new anti-angiogenic agents in details later.

The safety, feasibility and efficacy of anti-angiogenic therapies for patients with advanced malignant melanoma are currently under investigation in clinical phase I-III trials. The humanized monoclonal antibody Vitaxin, which is directed against the v3 integrin [59], an angiogenesis inhibitor thalidomide, combined with the cytotoxic agent temozolomide [60], recombinant TNF through isolated limb perfusion [61], and a ribozyme-based inhibitor targeting VEGF receptor-1 [62] are being tested in clinical trials for patients with progressed or metastatic melanoma. The combination of temozolomide and thalidomide was well tolerated and had antitumor activity in some patients with advanced melanoma [60]. A phase I trial of the angiogenesis inhibitor TNP-470, a derivative of fumagillin, resulted in the induction of a long-term, stable disease state in one patient with progressive metastatic melanoma [63]. Since malignant melanoma cells release a number of different angiogenic factors, a combination of these and other anti-angiogenic agents, combined with traditional or low-dose chemotherapy or with immunotherapy, might ultimately be needed to inhibit melanoma growth.

2.1.3. Endostatin

Endostatin is an endogenous collagen XVIII–derived angiogenesis inhibitor identified and purified from a murine hemangioendothelioma cell line [64] and later characterized in mice [65]. It corresponds to a 20-kDa fragment derived from the COOH-terminal NC1 domain of type XVIII collagen [64-67]. Recombinant endostatin efficiently blocks angiogenesis and suppresses primary tumor growth and metastasis in experimental animal models without any apparent side effects, toxicity, or development of drug resistance [54, 64,68,69].

New insights into the molecular mechanisms associated with endostatin inhibition of tumor growth are emerging. Recent studies have reported that endostatin interferes with FGF-2-induced signal transduction, blocking endothelial cell motility [70], inducing apoptosis [71], causing G1 growth arrest of endothelial cells through inhibition of cyclin D1 [72]. In addition, endostatin also blocks VEGF-mediated signaling *via* direct interaction with the VEGF-R2/KDR/Flk-1 receptor tyrosine kinase in human umbilical vein endothelial cells [73], and blocks TNF–induced c-Jun NH2-terminal kinase–dependent proangiogenic gene expression [74]. Endostatin rapidly down-regulates many genes in growing endothelial cells as well, including immediate-early response genes, cell cycle–related genes, and genes regulating apoptosis inhibitors, MAPKs, FAKs, and G-protein-coupled receptors mediating endothelial cell growth, mitogenic factors, adhesion molecules, and cell structure components [75]. Conversely, it was shown that endostatin up-regulates many anti-angiogenic genes in human microvascular endothelial cells. Endostatin also affects the signaling events that are not associated with angiogenesis, thus demonstrating the importance of inter-pathway communication in this signaling network [76].

Recombinant "immobilized" human endostatin interacts with $\alpha 5$ and αv integrins on the surface of human endothelial cells. Furthermore, this endostatin-integrin interaction is of functional significance *in vitro*, as immobilized endostatin promotes integrin-dependent endothelial cell functions [77]. Endostatin binds to the $\alpha 5\beta 1$ integrin and inhibits the migration of endothelial cells by blocking signaling pathways *via* Ras and Raf and further downstream *via* ERK1 or p38 [78]. Conversely, endostatin inhibits chemotaxis, without affecting the intracellular pathways known to regulate endothelial cell migration and proliferation/survival, without affecting phospholipase C-γ, Akt/protein kinase B, p44/42 MAPK, p38 MAPK, and p21-activated kinase activity [79]. Therefore, more work needs to be done to sort out the precise mechanism of endostatin action.

Endostatin binds to heparin [80] and with low affinity to all surface heparan sulfate proteoglycans that are involved in growth factor signaling [81-83]. The anti-angiogenic activity of endostatin seems to depend on the interactions with heparan sulfate proteoglycans, possibly by an interaction between discontinuous sulfated domains in heparan sulfate proteoglycans and arginine clusters on the surface of endostatin [84].

Endostatin inhibits the activation and activity of certain MMPs (i.e., MMP-2, -9, and -13 and MT1-MMP) and binds directly to at least MMP-2 and -9 [85-87]. In addition to MMPs, endostatin has been shown to interfere with the action of other proteases, like the plasminogen activator system [88]. Interestingly, certain MMPs can generate endostatin-containing peptides differing in molecular size (20-30 kDa) from human type XVIII collagen [89]. The physiologic levels of circulating endostatin in the serum are 40 to 100 ng/mL compared with the concentrations of endostatin (0.2-20 mg/mL) that are effective in the inhibition of tumor growth in various experiments. It has been shown that some of the anti-angiogenic and anti-tumor effects of endostatin might, in fact, represent high dose pharmacologic effects

which are not necessarily related to the physiologic function of endostatin [90]. In this regard, the physiologic levels of endostatin have little or no effect on the growth of fibrosarcomas and melanomas in collagen XVIII/endostatin knockout mice [91].

Three phase I trials have been published using recombinant human endostatin in a total of 61 patients with advanced metastatic cancer (including 11 melanomal cases) [92-94]. These studies administered daily endostatin doses of 15-600 mg/m^2/day by short intravenous infusion. No significant endostatin-related toxicity was noted. Endostatin displayed consistent linear pharmacokinetics with the area under the serum concentration-time curve reaching levels associated with activity in animal models, at doses of 300 mg/m^2/day. No formal disease responses were seen although some evidence of anti-neoplastic activity was noted with one patient with metastatic pancreatic neuroendocrine tumor experiencing a minor response [92]. Other administration regimens, such as daily subcutaneous injection of recombinant human endostatin have been also tried [95].

2.1.4. Thrombospondins (TSP)

Thrombospondin-1 (TSP-1) was the first protein to be recognized as a naturally occurring inhibitor of angiogenesis [96]. It is a large multifunctional ECM glycoprotein that regulates various biological events, like cell adhesion, angiogenesis, cell proliferation and cell survival, transforming growth factor-β (TGF-β) activation, and protease activation [97,98]. Some studies suggest that TSP-1 may possess dual activity (with both proangiogenic and antiangiogenic properties) depending on proteases that generate fragments of TSP-1 [99,100]. It has been shown to inhibit tumor growth and metastasis, thus making it a potent inhibitor of *in vivo* neovascularization and tumorigenesis. Overexpression of TSP-1 in mice suppresses wound healing and tumorigenesis, whereas the lack of functional TSP-1 results in increased vascularization of selected tissues [101-103]. Expression of TSP-1 was inversely correlated with malignant progression in breast and lung carcinomas and melanomas [104]. To evaluate the importance of TSP-1 in the progression of naturally arising tumors *in vivo*, Lawler *et al*. have crossed TSP-1-deficient mice with p53-deficient mice. In the p53-null mice, the absence of TSP-1 decreased survival. They also determined more directly whether host TSP-1 inhibited tumor growth by implanting melanoma and testicular teratocarcinoma cells into the TSP-1-null mice. The tumors grew faster on the TSP-1-null background and exhibited an increase in vascular density, a decrease in the rate of tumor cell apoptosis, and an increase in the rate of tumor cell proliferation [105]. The anti-angiogenic activity of TSP-1 has been mapped to the type 1 repeats and within the NH2-terminal portion of the molecule within the procollagen-like domain. TSP-1 and peptides from the type 1 repeat region (tryptophan-rich, heparin-binding sequences and TGF-β1 activation sequences) were evaluated in two models of retinal angiogenesis. TSP-1 inhibited angiogenesis in both experimental models, but peptides from the native TSP-1 sequence containing both the tryptophan-rich repeat and the TGF-β1 activation sequence or containing only the tryptophan-rich, heparin-binding sequence had distinct efficiencies in the two models. These results suggest that the type 1 repeats of TSP-1 contain two subdomains that might independently influence the processes of neovascularization [106]. The existence of two subdomains also explains how TSP-1 may block FGF-2 and VEGF angiogenic signals *via* two independent pathways [107]. TSP-1 is able to distinguish pathologic neovascularization from pre-existing vasculature due to the dependence of proliferating endothelial cells on Fas/Fas ligand (FasL)-mediated apoptosis. TSP-1 up-regulates FasL expression on endothelial cells. Expression of the FasL receptor, was low in quiescent endothelial cells but greatly enhanced by inducers of angiogenesis, thereby specifically sensitizing the stimulated cells to apoptosis by inhibitor-generated FasL [108].

ABT-510 is an angiogenesis inhibitor derived from TSP-1, a naturally occurring angiogenesis inhibitor. ABT-510 was administered subcutaneously in patients with advanced solid malignancies, to assess safety, pharmacokinetics, and serum markers of angiogenesis. Thirty-nine patients received a total of 144 treatment cycles. Stable disease lasting for six cycles or more was seen in six patients. ABT-510 demonstrated a low toxicity profile and linear, time-independent pharmacokinetics at biologically relevant plasma concentrations. The significant number of patients with prolonged, stable disease and the convenient dosing method merit further studies with this and possibly other angiogenesis inhibitors [109]. More recently the same group assessed ABT-510 with gemcitabine and cisplatin in patients with solid tumors including three progressed melanoma. Patients received a 3-week cycle of intravenous gemcitabine (1250 mg/m^2 on days 1 and 8) and cisplatin (80 mg/m^2 on day 1) in combination with ABT-510, which was administered subcutaneously twice daily at doses of 50 mg or 100 mg. One melanoma patient showed partial response [110].

TSP-2 also shows anti-angiogenic activity. Injection of TSP-2-transfected squamous cell carcinoma cells into the dermis of nude mice resulted in inhibition of tumor growth that was even stronger than the inhibition observed with TSP-1-transfected cells. The combined overexpression of TSP-1 and TSP-2 completely prevented tumor formation. Extensive areas of necrosis were observed in TSP-2-expressing tumors, and both the density and the size of tumor vessels were significantly reduced [111]. Furthermore, tumor angiogenesis was significantly enhanced in TSP-2-deficient mice. Although TSP-2 deficiency did not affect tumor differentiation or proliferation, tumor cell apoptosis was significantly reduced [112]. The anti-angiogenic role of TSP-2 was further confirmed with an implant system that continuously produces TSP-2. Fibroblasts, which overexpress TSP-2 are transplanted into nude mice resulting in increased levels of circulating TSP-2, inhibiting tumor growth and angiogenesis of human squamous cell carcinomas, malignant melanomas, and Lewis lung carcinomas implanted at a distant site [57]. It has been shown recently that the anti-angiogenic region of TSP-2 lies approximately within the 80-kDa fragment of the NH2-terminal globular region [113]. Daily injections of TSP-2 resulted in a significant inhibition of the growth of human squamous cell carcinomas *in vivo* and reduced tumor vascularization. Possible mechanisms for this anti-angiogenic activity include the inhibition of VEGF-induced endothelial cell migration, vessel tube formation, and/or increased endothelial cell–specific apoptosis [113].

2.1.5. Other Reagents

EMD 121974, an antagonist of αvβ3 and αvβ5 integrin, produced clinical benefits in melanoma patient preclinical studies. A phase I trial demonstrated good tolerance [114]. Another humanized IgG1 immunoglobulin (Medi-522) targeting the αvβ5 integrin was tested in a phase I study which accrued 19 patients. Tolerance was acceptable and 7 patients achieved prolonged SD with 2 lasting more than 9 months [115].

A specific Raf-1 inhibitor was developed termed BAY 43-9006. This BRaf inhibitor, BAY 43-9006 (sorafenib) has shown encouraging results when administered together with chemotherapy and is now being assessed in randomized studies. This evaluation study included 35 participants in Phase I/II studies, the majority who had advanced metastatic melanoma. Of the 35 participants, fourteen participants (40 percent) demonstrated tumor shrinkage of 50 percent or greater, which lasted for six months or more [116].

2.2. Pigment Epithelium-Derived Factor (PEDF)

2.2.1. Characterization of PEDF

PEDF, a glycoprotein that belongs to the superfamily of serine protease inhibitors, was first purified from human retinal pigment epithelial cell conditioned media as a factor with potent human retinoblastoma cell neuronal differentiation activity [117]. Recently, PEDF has been shown to be a potent inhibitor of angiogenesis in both cell culture and animal models. Indeed, PEDF is reported to inhibit retinal endothelial cell growth, migration and suppress ischemia-induced retinal neovascularization [118,119]. Furthermore, loss of PEDF was associated with angiogenic activity in proliferative diabetic retinopathy [120]. However, a functional role for PEDF in tumor growth and angiogenesis remains to be elucidated.

PEDF possesses several physiologic properties that make it a potentially important protein in the regulation of angiogenesis, in neuronal cell survival, in maintaining self-renewal of neural stem cells microenvironmentally, and in the protection of neurons from neurotoxic agents. Its anti-angiogenic activity is selective, in that PEDF targets only new vessel growth but spares existing vessels, and its action is reversible. It is a protein that is highly up-regulated in the G0 cell cycle phase of early-passage G361 cells compared with rapidly proliferating or senescent cells and thus it is also linked to both the control of the cell cycle and cell senescence [121,122].

A recent study highlighted two beneficial aspects of PEDF treatment on tumor growth and expansion. One is the direct suppression of tumor angiogenesis in several ways. PEDF is strongly expressed in normal murine kidney, and the loss of angioinhibitory activity here may contribute to increased pathologic angiogenesis in Wilms' tumors [123]. In addition, PEDF may serve as a multifunctional anti-tumor agent in neuroblastomas, not only inhibiting angiogenesis but also increasing the numbers of Schwann cells and differentiated tumor cells that in turn can produce PEDF [124]. A loss of PEDF expression was also detected in glioma tumor progression [125]. In PEDF-deficient mice, there were increased numbers of stromal vessels associated with epithelial cell hyperplasia [126]. Another PEDF activity includes the capability to induce of FasL-dependent apoptosis in tumor cells. PEDF up-regulates endothelial cell FasL. Expression of an essential partner of FasL, the Fas/CD95 receptor, is usually low on quiescent endothelial cells but is greatly enhanced after angiogenic induction, thereby specifically sensitizing the stimulated cells to an apoptotic signal by PEDF-generated FasL. The antiangiogenic activity of PEDF, both *in vitro* and *in vivo*, was dependent on this dual induction of Fas and FasL and the resulting apoptosis [58,107].

PEDF can be given therapeutically as a soluble protein or by viral-mediated gene transfer [127,128]. It is stable and nontoxic when injected systemically. PEDF gene transfer suppresses tumor vascularization and growth while prolonging survival in syngeneic murine models of thoracic malignancies. Gene transfer of PEDF using adenoviral-associated vectors also inhibited ischemia-induced neovascularization [129].

A recent study also suggests that, in physiologic conditions, a critical balance between PEDF and VEGF exists, and that PEDF may even counteract the angiogenic potential of VEGF. Under oxidative stress, PEDF levels decrease, disrupting the angiogenic balance [130]. This critical balance between PEDF and VEGF is important to prevent the development of choroidal neovascularization [131,132]. In addition, bone angiogenesis and matrix modeling may also be mediated by the dynamic interplay between both PEDF and VEGF [133].

2.2.2. Efficacy of PEDF for Melanoma Growth Inhibition

We have recently reported [58] that several human melanoma cells expressed substantial amounts of PEDF, and the expression levels of PEDF in these tumor cells were comparable with that of normal human cultured melanocytes. In contrast, expression levels of VEGF among these cells were quite variable; melanoma cells were characterized by a strong expression of VEGF, while little VEGF protein was detected in cell lysates from normal melanocytes. These observations suggest a pathological role for tumor VEGF production in the development of malignant melanoma [125]. A decrease or loss in PEDF production by tumor cells themselves might not contribute to the initiation or progression of the malignant melanoma tumor. However, we, together with others, have recently shown that PEDF protected retinal vascular and neuronal cells from oxidative stress-induced injury [134,135]. H_2O_2 is formed as a byproduct of melanin synthesis, following ultraviolet irradiation in mammalian skin [136]. Taken these results into consideration, PEDF secreted by melanocytes might be involved in the natural maintenance of normal skin homeostasis through its anti-oxidative properties, although we do not fully understand the physiological roles of PEDF in melanocytes in skin [136]. High levels of PEDF in normal cells may also inhibit the formation of oxidative stress related mutagenic free radicals that can cause DNA damage and neoplastic initiation and tumor progression.

Overexpression of PEDF was found to decrease tumor angiogenesis and almost completely inhibits the growth of melanoma xenografts in nude mice. Therefore, the inhibition of tumor angiogenesis by PEDF may be a promising ap-

proach for melanoma treatment. Furthermore, *in vitro*, PEDF dose-dependently retarded growth and induced apoptotic cell death in melanoma cells, which was completely blocked by treatments with a neutralizing antibody against FasL. These results suggest that PEDF directly elicits apoptosis in melanoma cells in a FasL-dependent manner. Taken together, our study has also highlighted two beneficial aspects of PEDF effects in melanoma growth and expansion; one is the suppression of tumor angiogenesis, and the other is induction of Fas L-dependent apoptosis in tumor cells. PEDF is therefore a promising novel therapeutic agent for the treatment of patients with certain types of cancer including melanoma.

Malignant melanoma responds well to anti-angiogenic therapy using other endogenous angiogenic inhibitors such as angiostatin (plasminogen kringles 1-4) and endostatin [137,138]. Since plasminogen kringle 5 was recently reported to inhibit ischemia-induced retinal neovascularization in a rat model by down-regulating VEGF and up-regulating PEDF [139], the anti-angiogenic and growth inhibitory effects of angiostatin on melanoma cells might work in a similar manner and therefore could be ascribed, at least in part, to changes in PEDF activity.

Lastly, we will discuss the biologically relevant serum concentrations of PEDF that might exert anti-tumor effects on malignant melanoma *in vitro*. Petersen *et al.* recently reported that the estimated human blood concentration of PEDF was about 100 nM [140]. These observations suggest that at the physiologic concentrations of PEDF seen in humans might have anti-tumor effects. Since hypoxia and cytokines down-regulate PEDF expression levels in various cell types [118,134,141], a decrease or loss in PEDF production might contribute to the likelihood of tumor growth and expansion *in vivo*. Recently, serum concentrations of PEDF were found to be decreased in patients with hepatocellular carcinoma compared to healthy volunteers and patients with chronic hepatitis, further supporting this speculation [142].

3. METASTASIS AND THERAPEUTIC STRATEGIES AGAINST METASTASIS

3.1. Mechanism of Metastasis

The existence of invading cancer cells in an organism does not necessarily imply metastases and a fatal outcome [9]. Invasion is certainly a prerequisite for metastasis, e.g. without invasion metastasis cannot take place [143-146]. There are many steps in the cascade from the initiation of melanoma cancer to metastasis: multiple initiation events → growth → angiogenesis → progression → tumor cell selection → detachment → adhesion to the basal membrane → destruction of the basal membrane → motility → adhesion at the basal membranes of vessels → migration through the vessel wall → survival in the vessel and embolization → passage through the vessel wall and entry into metastatic target organs → local factors → invasion and growth of metastasis in target organ [9].

Firstly, acquired genetic susceptibility enables the stepwise selection of variant subclones of cancer cells. In a minority of cells, loss of cell-cell adhesion will occur during growth. Proteins (e.g. receptor for advanced glycation end products (RAGE)) and amphoterin have been identified as a receptor-ligand pairs in molecular checkpoints that regulate survival, invasiveness, growth and spread of tumor cells [147]. Regulation of the molecular events necessary for invasion involves a spatial and temporal coordination, of cyclic on-off processes (at the level of individual cells) and motility coupled with controlled adhesion to the extracellular matrix, which allows an invading cell to move through the three-dimensional matrix. Several gene families are involved in invasion: matrix metalloproteinases [148], urokinase plasminogen activator/ receptor [149], integrins [150], cathepsins [151] and many others, most of which are presently unidentified. The functions of proteins encoded by these genes are the regulation of tumor cell adhesion to each other as well as of tumor cells and the ECM, the synthesis of proteases, migration of tumor cells, cytoskeletal remodeling and the synthesis of new ECM components (ECM remodeling). In a tumor, the close proximity of neoplastic and non-neoplastic cells (e.g. fibroblasts, pericytes and inflammatory, endothelial and myoepithelial cells) affects the microenvironment of the ECM [152]. The microenvironment is modified and remodeled in part by proteases. Notably, the host stroma is responsible for most of the increase in protease production, and the cellular origins of the proteolytic machinery vary in different tumor types. A recent finding is that intratumoral hypoxia is correlated with an increased risk of invasion probably due to the selection of mutated proteins that harbor invasive properties and by the expression of genes, the products of which promote invasion mediated by hypoxia-inducible factor 1 (HIF-1) [153]. A new finding is that the breakdown of epithelial cell homeostasis leading to aggressive cancer progression has been correlated with the loss of epithelial characteristics and the acquisition of a migratory phenotype [146]. This phenomenon, referred to as epithelial-mesenchymal transition (EMT) is considered a crucial event in late-stage tumorigenesis [154]. A multitude of EMT models have been developed in different tissues. EMT is accompanied by loss of epithelial glycoprotein 2 (MOC-31) [155], upregulation of MMPs [156] and increased expression of N-cadherin [157]. Recently, the diversity of molecular mechanisms contributing to the plasticity of epithelial cells has been studied. It has become evident that tumor metastasis and angiogenesis are intrinsically related [9]. A further crucial element both in cancer invasion and metastatic outgrowth is the interaction between tumor cells and stroma [158]. Conventional wisdom has assumed that invasion and metastasis are late events. Our present knowledge suggests that invasion can be either an early or clinically dormant event.

3.2. Chemokine in Metastasis

Chemokines are chemotactic cytokines that cause the directed migration of leukocytes, and are induced by inflammatory cytokines, growth factors and pathogenic stimuli [159-161]. Chemokine signaling results in the transcription of target genes that are involved in cell invasion, motility, interactions with ECM and cell survival [162]. Chemokine signaling can coordinate cell movement during inflammation, as well as homeostatic transport of hematopoietic stem cells (HSCs), lymphocytes and dendritic cells. Directed migration of cells that express the appropriate chemokine receptor occurs along a ligand chemical gradient allowing cells

to move towards high local concentrations of chemokines. More than 50 chemokines have been discovered so far and there are at least 18 human seven-transmembrane-domain chemokine receptors [163]. In general, these receptors, which belong to the G-protein-coupled receptor family, bind to more than one type of chemokine. The profile of chemokine-receptor expression on an individual cell is determined by its lineage, stage of differentiation, and microenvironmental factors such as chemokine concentration, the presence of inflammatory cytokines and hypoxia. The pattern of chemokine receptor and ligand expression in a tissue generally correlates with the numbers and types of infiltrating cells that are typically present. The chemokine gradient that attracts infiltrating cells can be created by different cell populations in a tissue. During infections, the first cells that produce chemokines are probably tissue leukocytes, but fibroblasts, endothelial cells and epithelial cells (both normal and malignant) are also able to produce chemokines and generate a chemokine gradient. Although originally identified on leukocytes, functional chemokine receptors are also found on endothelial cells [164] and on some epithelial cells, particularly those that have been malignantly transformed [165-167]. Many human cancers have a complex chemokine network that influences the extent and phenotype of this infiltrate, as well as tumor cell growth, survival, migration and angiogenesis [168]. It has been shown that there is a significant chemokines influence on the metastatic potential and site-specific spread of tumor cells.

Recent reports imply that malignant melanoma cells also express various chemokines in association with tumor progression and metastasis [169]. CXCR3 and CXCR4 receptors are expressed in progressed melanoma cell line BLM. The ligands for CXCR3 and CXCR4 activate various GTPases, causing chemotaxis and modulation of melanoma cell adhesion to fibronectin [170]. Clinically, melanoma patients with highly expressed CXCR3 and/or CXCR4 tumor cells have statistically poor prognostic signs such as ulceration, increased tumor thickness and lymphatic infiltration [171,172], although there is a controversy as to CXCR3 [171]. Risk of metastasis are also substantially increased when melanoma cells express CXCR4 [173-175]. Another chemokine, CCR10 expression was also found in melanoma and it was directly correlated with the tumor depth and sentinel lymph node metastasis [176]. Although there have been no clinical evaluation, CCR7 is indicated an association with lympho node metastasis *in vitro* [169,177], and CCR9 with small intestine metastasis [178].

3.3. Novel Therapeutic Strategies to Block Metastases of Tumors

The main cause of treatment failure and death in cancer patients is metastasis - the formation of secondary tumors in organs distant from the original neoplastic cell tissue. Adjuvant therapy of proven efficacy is not currently available for cancer patients, therefore the search for new targets for therapeutic reagents is required to prevent both proliferation and metastases.

It is clear that chemokines and their receptors are involved in malignant progression and that a better understanding of chemokine signaling in this process could lead to new therapeutic strategies for cancer. With this in mind, it is possible that drugs that are being tested in inflammatory and autoimmune diseases that target the chemokine network [179] could also be useful as cancer biotherapies. As the chemokine network is both large and complex, it is unlikely that an individual chemokine antagonist would be powerful enough to inhibit cancer cells, and inhibitors of chemokine-inducing cytokines, such as TNF-α, could also be useful. Chemokine and cytokine antagonists have the potential to inhibit tumor-promoting leukocyte infiltrates, metastatic spread and angiogenesis [180,181].

Recent preclinical studies have reported chemokine-receptor antagonist anticancer activity in several murine cancer models. For example, tumor cells express CCL5, and the CCL5 receptors CCR1 and CCR5 are expressed by the leukocyte infiltrate. Daily treatment of tumor-bearing mice with the CCR1 and CCR5 antagonist, Met-CCL5 [182], led to modest anticancer effects at doses similar to those that have activity in animal models of inflammatory disease. Specifically, the total number of inflammatory cells and the proportion of infiltrating macrophages decreased in tumors treated with Met-CCL5 [183]. Inhibiting tumor cell chemokine-receptor signaling has the potential to induce growth arrest or apoptosis, and prevent invasion and metastasis [184,185]. Antibodies to CXCL12 also inhibit organ metastases of non-small-cell lung cancer cells when concurrently administered with tumor cell injection into mice [186]. Systemic administration of the CXCR4 antagonist AMD3100 inhibited the growth of intracranial glioblastoma and medulloblastoma xenografts, and increased tumor cell apoptosis within 24 hours [187]. Furthremore, more recently, daily subcutaneous injection of microcapsules containing a CXCR4 antagonist (4F-Benzoyl-TE14011) suppressed pulmonary metastasis in murine melanoma model [188]. Therefore, CXCR4 is a potential therapeutic target in human cancer, although more extensive studies using better established tumors and antagonists of other receptors are required. Where there is high expression of CXCL12 at the site of the primary tumor, it is important to ensure that antagonists, such as AMD3100, do not encourage tumor cell release, thereby increasing, rather than decreasing, the risk of metastasis. Finally, as has been shown in many experimental cancer studies, an alternative approach is to manipulate the cancer chemokine network to encourage the influx and activation of host immune cells that have general or specific tumor-destructive capacities.

3.4. Advanced Glycation end Products (AGEs) – Receptor for AGEs (RAGE) Interaction

Advanced glycation end products (AGEs), non-enzymatically glycated protein derivatives, were originally thought to accumulate in various tissues and have been implicated in the development of diabetic vascular complications, e.g. retinopathy and nephropathy [189,190]. Recent studies demonstrated that the formation and accumulation of AGEs progress at an accelerated rate intra- and extracellularly after the generation of oxidation stress [191]. The receptor for advanced glycation end products (RAGE), a multi-ligand member of the immunoglobulin superfamily of cell surface molecules, interacts with distinct molecules implicated in homeostasis, development and inflammation [192]. RAGE binding by ligands such as AGEs triggers the activation of

key cell signaling pathways, thereby reprogramming cellular properties. Recently, Taguchi and colleagues have identified RAGE as a molecular checkpoint that regulates not only the invasiveness but also the growth and movement of glioma cells [147]. However, the complete role of RAGE is still unclear in melanoma proliferation and metastasis.

Recently, we demonstrated that RAGE was expressed in human malignant melanoma cells. Furthermore, glyceraldehyde-derived AGE (AGE2) and glycolaldehyde-derived AGE (AGE3) enhanced proliferation, migration and invasion of this malignant melanoma cell line *in vitro* [193]. Finally, treatment by intraperitoneal injection of the neutralizing anti-RAGE antibody reduced tumor formation, prolonged survival rate and inhibited lung metastases in nude mice [193].

It has been reported that RAGE is expressed by a range of cell types, including endothelial cell, astrocytes, and some malignant cells such as malignant glioma and squamous cell carcinoma, contributing to homeostasis, development, inflammation, and carcinogenesis [191,194-196]. We and other groups have reported that AGEs were present in various tissues including blood vessel endothelial cells, mesenchymal cells, and as part of the ECM [197,198]. Accumulation of AGEs initiated various important processes including angiogenesis in diabetic microangiopathies [194]. AGE formation in the ECM of skin is accelerated by ultraviolet-induced oxidation [198] and AGEs generate active oxygen species in the skin during ultraviolet irradiation [199] suggesting the presence of a vicious cycle of AGEs formation. Furthermore, AGEs can activate RAGE expression causing enhanced AGEs-RAGE interaction [200]. It is thus hypothesized that activated cells that include not only tumor cells but also stromal cells located in peri-tumor sites, synthesis AGEs. Taken together, we hypothesis that melanoma cells proliferate and invade with abundant accumulation of extracellular AGEs in skin.

We have highlighted the pathways of AGEs formation and characterized distinct AGEs classes (AGE1-5) with different cell mediated responses [201-203]. In the present study, AGE2 and AGE3 but not other AGEs, can up-regulate melanoma cell growth, migration, and invasion *in vitro*. Recently, we also reported strong RAGE binding by AGE2 and AGE3, but not other AGEs [204]. Taken together, AGE2- and AGE3- RAGE interactions have an important role in the progression of melanoma. Although previous papers reported that CML-RAGE interaction mediates cell signaling [205], our data showed CML has little effective influence on melanoma proliferation, migration, and invasion. We speculate this disparity is dependent on cell transformation state (malignant or normal) or cell type.

We also assessed the therapeutic efficacy of AGEs-RAGE interactions in a xenograft model. In our experiments, blocking RAGE by systemic administration of neutralizing RAGE antibody significantly inhibited the growth of G361 xenograft tumors and spontaneous lung metastasis. Furthermore, the inhibition of RAGE prolonged the survival of G361 tumor-bearing mice. Previously we reported that this interaction could play a significant role in the progression of pancreatic cancer through the induction of autocrine platelet-derived growth factor-B [195]. Furthermore, Taguchi *et al.* showed that this interaction regulates not only the growth but also the movement and invasiveness of glioma tumor cells [191]. In addition, the presence of AGEs was confirmed in human melanoma beds, whereas AGEs were hardly detected in normal skin, suggesting the interaction of up-regulated RAGE in melanoma cells with AGEs which are present in tumor beds to promote melanoma progression. We concluded that these interactions regulate various malignant tumors, which have a particularly high invasive and metastatic potential.

CONCLUSION

Only cells from malignant tumors invade surrounding tissues and travel to distant organs. It was thought that invasion and metastases were late events in the clinical course of a patient's cancer. However, we now know that invasion can be both early and clinically 'silent'. An understanding of the molecular basis for this aggressiveness aims to lead to therapies that block the transition of a tumor from benign to malignant, and maintain the disease locally.

In addition, there is now significant evidence to indicate that the induction of angiogenesis is an important stage for many tumors, especially melanoma progression. As an increasing number of angiogenesis inhibitors are being tested on other tumor types, these trials should be expanded to include melanoma patients with progressive disease. Relative systemic levels of proangiogenic and antiangiogenic factors likely govern tumor progression by regulating the tissue "angiogenic balance." Conversion of dormant carcinomas to invasive malignant carcinomas is considered to involve a shift in favor of enhanced angiogenesis potential. Influenced by oncogenes and tumor suppressor genes, disruption of the "angiogenic checkpoint" *via* increases in angiogenic factors, such as VEGF, or decreases in the physiologic levels of endogenous inhibitors of angiogenesis, like PEDF, may represent an important step in the progression of cancer.

One of the most pressing clinical questions is to determine whether it is best to exclusively use these new anti-angiogenic agents or to combine them with conventional cytotoxic drugs so that the survival of patients can be significantly increased [206]. This challenge implies a need for new clinical development models that preferably consider the cytostatic rather than the cytotoxic nature of anti-angiogenic agents, in addition to the possibility of prolonged therapy with these agents and the rationale for combining them with other cytotoxic therapies. Ongoing clinical trials are applying these concepts with the prospect of using these anti-angiogenic therapies in clinical practice.

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