THE CONTRIBUTION OF VASCULAR ENDOTHELIAL GROWTH FACTOR INDUCED ANGIOGENESIS TO THE PROGRESSION OF PRIMARY HUMAN MELANOMA

by

Liisa Ann Bayko

A thesis submitted in conformity with the requirements for the Degree of Master of Science, Graduate Department of Medical Biophysics, University of Toronto

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ABSTRACT

"The Contribution of Vascular Endothelial Growth Factor Induced Angiogenesis in the Progression of Primary Human Melanoma"

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Early stage primary human melanomas are known to remain relatively avascular and dormant for up to a decade or longer after which they may give rise to more rapidly growing, vascular and metastatically-competent primary tumors. The clinical behavior of melanoma recapitulated experimentally by injection of cell lines established from such tumors into nude mice. WM1341B cells, which were isolated from early stage human melanoma, are non-tumorigenic and display a dormant phenotype in nude mice. These cells produce little or no vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis, in culture. In order to determine whether their in vivo dormant behavior may be related to an inability to induce tumor angiogenesis, subpopulations of WM1341B cells were engineered to constitutively overexpress the VEGF121 isoform. This modification was sufficient to induce overtly progressively growing tumors by several independent VEGF121 producing clones and could be strongly suppressed by treatment of mice with a monoclonal anti-VEGF neutralizing (A4.6.1) antibody. These results support the notion that defective angiogenesis may, in part, account for the dormant phenotype of early stage primary melanoma lesions. Furthermore, this system may be useful as a tool to screen new anti-angiogenic agents for cancer therapy.
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Summary

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CHAPTER 1

INTRODUCTION
Circa 400 B.C. Hippocrates likened the long distended veins radiating from some tumors to the limbs of a crab (1). Centuries later the crucial role of angiogenesis—the growth of new blood vessels from pre-existing vessels—in the development and progression of cancer is only beginning to be appreciated. Globally, the WHO estimates that cancer kills roughly six million people annually (2). Furthermore, of the more than 100 forms of the disease, the incidence of malignant melanoma is increasing at a faster rate than any other cancer in the United States, Australia, northern Europe, and Canada, with the exception of lung cancer in women (3). The worldwide increase in incidence of melanoma, coupled with the lack of an effective treatment for advanced stages of the disease, necessitates further exploration of a number of important biological questions regarding melanoma development and progression. In this regard, because solid tumors are known to be angiogenesis dependent (4,5), further investigation into the role of angiogenesis in melanoma progression may have significant implications for our understanding and treatment of this disease.

**Melanoma progression**

Malignant melanoma is a highly malignant form of skin cancer which originates from cells of the melanocytic lineage. Melanocytes are neural crest derived cells that migrate to the basal layer of the epidermis where they synthesize melanin pigment in order to protect the skin from ultraviolet radiation (6). Clinical and histopathological observations suggest that the evolution of malignant melanoma from normal melanocytes to metastatic disease occurs through multiple progressive stages (7).
Melanocytes, residing in the epidermal-dermal interface, can form a focus of cytogenetically normal cells called a common nevus (8). Most melanocytic nevi slowly regress and eventually disappear over time. This regression has been thought to be the result of an active immune response or a differentiation pathway which leads to cell senescence (3). The first pathological sign of impending malignant disease is architectural and cytological atypia which is termed dysplastic nevi (8). Although progression of melanocytic nevi to malignant disease is a relatively rare event, a small proportion of these precursor lesions may eventually give rise to the first stage of malignant melanoma, namely the radial growth phase (RGP). RGP consists of a slow growing lesion which expands outward in a radial manner (7,9) reaching a size often greater than 10 mm in horizontal diameter (3). While RGP growth is confined mainly to the avascular epidermis, occasionally these lesions may invade into the underlying dermis. This is called ‘invasive’ RGP or melanoma ‘in situ’. It is generally believed that tumor cells in RGP lesions are metastatically incompetent. As such, primary RGP lesions are almost always cured by surgical excision of the lesions (6).

Although RGP lesions can remain in this indolent stage for years, such lesions can evolve over time to become rapidly growing, vertical growth phase melanoma (VGP) (9). VGP lesions grow in a vertical manner extending beyond the epidermis into the underlying dermis. Initially, melanoma cells are unable to grow in the microenvironment of the dermis and therefore some of the infiltrated tumor populations regress (9). Eventually, some of the cells develop resistance to the growth inhibitory factors in the dermis and can thus expand vertically (9). VGP lesions are thought to contain a new subclone of tumor cells which are capable of proliferating in the foreign environment of the underlying dermis, some of which may be metastatically competent (10).
During this invasive stage of growth, tumor cells gain access to both blood and lymphatic vessels which are located in the dermal mesenchyme. This proximity to the vasculature may have several implications for melanoma progression including the potential for the tumor cells to metastasize.

The final stage of melanoma progression is the formation of distant metastases. Melanoma can metastasize to any organ, but the lymph nodes, skin, lung, brain, and liver represent the most common initial sites of metastatic melanoma (3).

**Genetic alterations associated with melanoma progression**

Although the precise etiological factors leading to the development and subsequent progression of human melanoma remain poorly understood, there is some epidemiological evidence suggesting a strong role for sunlight, particularly in the UV-B range in the development of the disease (6). Ultraviolet light is known to cause DNA damage in melanoma cell lines, although direct evidence for the sunlight as a direct causative factor in melanoma development has yet to be demonstrated. Aside from environmental influences such as sun exposure, susceptibility to melanoma can be inherited. Approximately 10% of human melanoma is familial in nature (3). This suggests a role for genetic alterations in the development of the disease.

Although the dynamics of progression vary among different tumor types, the sequential stages of cutaneous melanoma progression suggest that the melanocyte must accumulate multiple genetic alterations to progress from one stage of the disease to the next and ultimately to the complete transformed (and malignant) phenotype. However, in contrast to certain tumors such as human colorectal carcinomas, the precise underlying genetic changes that are associated with
melanoma development and progression are not as well documented. However, strong evidence suggests that genes on chromosome 1, 6, 7, 9 and, to a lesser extent on 2, 3, and 10 may be involved in the pathogenesis of melanoma (8). These genetic alterations may eventually provide clues to the location of oncogenes and tumor suppressor genes involved in melanoma development and progression. Although a consistent pattern implicating any gene to melanoma has failed to emerge, mutations and altered expression of a variety of genes including EGF-R, N-ras, H-ras, p16 in familial melanoma, p53, nm23, and p21 (6,11-18), amongst others have been implicated as contributing to the development and progression of melanoma.

Molecular and biological alterations in melanoma progression

Aside from oncogenes and tumor suppressor genes, numerous studies, mainly correlative, have documented an array of molecular alterations in the expression and function of growth factors, cytokines, transcription factors, tyrosine kinases, proteolytic enzymes, angiogenic factors, chemotactic factors, integrins, and adhesion molecules accompanying the transition from normal melanocyte to metastatic melanoma.

In culture, normal melanocytes can only proliferate in the presence of exogenously added growth factors. Malignant melanoma cells on the other hand have acquired the ability to proliferate in the absence of exogenous growth factors. This 'growth factor independence' reflects the autocrine production of growth stimulators and cytokines by the melanoma cells, several of which have been identified (19). One of the major growth factors for melanoma cells is basic fibroblastic growth factor (bFGF) which acts as an autocrine growth factor for melanocytes which have progressed to the benign nevus and metastatic stages (20). Other
autocrine growth factors in melanoma include melanocyte growth stimulating activity (MGSA), nerve growth factor (NGF), platelet derived growth factor (PDGF), transforming growth factor alpha (TGF-α), and insulin growth factor-1 (IGF-1) (19,21) amongst others.

Another cytokine which may be important in melanoma progression is interleukin-6 (IL-6) which it is produced by stromal cells such as endothelial cells and fibroblasts. IL-6 has been shown to be growth inhibitory for melanoma cells in the early stages of progression i.e. RGP and early VGP (22). However, as these cells progress to more advanced stages they become resistant and may even be stimulated by IL-6 (22). A similar pattern has been seen with other growth factors such as of TGF-α, TGF-β, IL-β, oncostatin M, and tumor necrosis factor (TNF-α) (23). As such, the term 'multicytokine resistance' has been used to describe the tendency of cells from advanced melanoma to develop resistance, and in some cases mitogenic activity, in response to a number of different growth inhibitors. The combined phenotypes of 'multicytokine resistance' and 'multigrowth factor independence' may provide melanoma cells with the ability to grow in both in the dermal mesenchyme of the primary lesion and in the foreign environment of the organ(s) to which the melanoma metastasizes (21).

The capacity of melanoma cells to invade a variety of tissues and extracellular matrices may, in part, be due to the repertoire of adhesion molecules they express on their cell surface. Recent investigations have reported changes in the expression of various adhesion molecules in the progression of melanoma from in situ to invasive and metastatic tumors including the upregulation of the integrins αβ3, α3β1, α4β1, and αβ1, downregulation of αβ1, and enhanced expression of intercellular adhesion molecule-1 (ICAM-1), MUC 18, and CD44 (24). The αβ3 integrin is expressed in metastatic melanoma but not in melanocytes, nevi and horizontally
spreading primary melanoma (25). This integrin, also known as the vitronectin receptor, enables melanoma cells to attach to a variety of extracellular matrix components which contain the RGD peptide sequence (Arg-Gly-Asp) (25). Thus $\alpha_\beta_3$ can bind to vitronectin, von Willebrand factor, thrombospondin, osteopontin, fibronectin and laminin. Interestingly, $\alpha_\beta_3$ is also required for angiogenesis (26). In an experimental system, a monoclonal antibody to $\alpha_\beta_3$ blocked angiogenesis induced by bFGF, TNF-$\alpha$, and human melanoma fragments (26).

Thus, there are a repertoire of molecular changes which accompany the progression of human melanoma from the most benign to the malignant stage of the disease. However, the mechanism by which any one of these particular changes contributes to progression and what genetic alterations lie behind these changes remain unresolved questions.

**Prognostic indicators of melanoma**

Despite the observation that many molecular changes accompany melanoma progression, the use of such changes as prognostic indicators has not been particularly successful. However, there are other variables which have a predicative value and some have been used in improving our ability to make a more accurate prognosis.

Clark (9) has established a classification system which utilizes the depth of invasion of the primary melanoma lesion (figure 1). Clark defines level I melanoma as a tumor which has its growth restricted to the epidermis, reminiscent of RGP melanoma, and level II melanoma as a tumor which has the ability to invade the loose papillary dermis, often referred to as 'thin' VGP. Melanomas which are classified as level I and II melanomas rarely give rise to metastases and are associated with an excellent prognosis, with 10 year survival rates of greater than 95%. On the
other hand, melanoma lesions described by Clark as level III or greater, have a marked tendency
Fig. 1 A schematic illustration of the anatomy of the skin demonstrating the locations of different levels of invasion using the Clark microstaging criteria (taken from Balch, C. M., and Milton, G. W. Cutaneous Melanoma. Clinical Management and Treatment Results Worldwide. L. B. Lippincott Co., 1985).
to metastasize via the vascular or lymphatic pathways. Patients in these groups generally have a much less favorable prognosis. For patients with melanoma 3.5 mm in thickness, the survival rate drops to only 33% to 58% over a ten year period.

To date, the best prognostic indicator for primary melanoma is Breslow's thickness classification system (27), in which vertical tumor thickness is measured from the top of the tumor to the deepest point of tumor penetration. In Breslow's system, 0.76 mm in thickness is considered to be the crucial dermal level, i.e. the trend appears to be that patients having a cutaneous melanoma which is less than 0.76 mm in vertical thickness have a very good prognosis and are usually cured by surgical removal of the tumor, whereas while those who present with lesions that are greater than 0.76 mm in thickness are more likely to develop metastatic melanoma and eventually succumb to the disease. Thus, it should be pointed out that a relatively small change in tumor size can have drastic implications in terms of prognosis and survival. This difference in patient outcome may be a consequence of 'qualitative' changes which accompany such small 'quantitative' differences, i.e. the proportion of metastatically competent melanoma cells populating "thin" lesions may be very low while the proportion of such cells can increase significantly as these lesions increase in size. This is the so-called "clonal dominance" hypothesis put forward by Kerbel and colleagues (10).

The relationship between melanoma and tumor vascularity

Although tumor thickness is currently the most reliable prognostic factor for cutaneous melanoma, exceptions do exist. For instance, a subset of thin melanomas metastasizes whereas some thick lesions do not (28). As a result, other parameters of melanoma progression have been
assessed in terms of their prognostic value. One potential prognostic indicator of melanoma is the use of tumor vascularity which has been gaining clinical importance because of its involvement in melanoma progression. A statistically significant increase in small blood vessels was found at the base of primary malignant melanoma and in melanoma metastases compared to common melanocytic nevi, suggesting that growth of malignant melanoma is closely related to the formation of blood vessels from the surrounding connective tissue, at least in the B16 murine melanoma cell line (29). It has also been shown that there is a gradual rise in vascularity with tumor progression in the melanocytic system with the formation of blood vessels beginning during the RGP stage (30).

Vascularization in human melanoma was found to be correlated with tumor thickness (31). In melanoma, detectable blood vessels has been found in lesions approximately 0.5-1 mm thick (31,32). Srivastava et al. (33) reported that there are prognostically significant differences in vascularity in melanocytic lesions classified as 'intermediate', ranging from 0.76-4.0 mm. Smolle et al. (34) reported similar findings and concluded that vascularity is closely related to proliferative activity in malignant melanoma. Fallowfield and Cook (32) reported a significant relationship between tumor thickness and percentage vascular volume and suggested that low tumor vascularity could correlate with a relatively favorable outcome in cutaneous melanoma, even in thicker lesions. Furthermore, a report published by our laboratory showed that in thin melanoma, defined as lesions less than 0.76 mm in depth, high intratumoral microvessel density was associated with a greater chance of metastases and death (28). Taken together, these results strongly suggest that extent of vascularity may be useful as an independent prognostic indicator in human melanoma.
On the other hand, some researchers have failed to find an association between extent of vascularization and prognosis in melanoma. Among them, Cornochan et al (35) found that for melanoma lesions in the range of thickness of 0.85-1.25mm, tumor vascularity does not have prognostic significance. Furthermore, Busam et al. (36) observed that in the case of thin melanoma, microvessel number does not have prognostic significance. The discrepancy between the aforementioned reports may be attributed to the use of small sample sizes and varying methods of blood vessel assessment in the studies. Thus, although there is strong evidence to suggest that increased vascularity may facilitate tumor growth in melanoma, the use of degree of vascularization (i.e. the number of vessels in so called “vascular hotspots”) as an independent prognostic indicator in solid tumors remains controversial.

**Tumor angiogenesis**

Angiogenesis is a vital component of many physiological and pathological processes including wound healing, maturation of corpus luteum, chronic inflammation, delayed hypersensitivity, as well as malignant disease. Whether prompted by physiological or pathological stimuli, the multistep process of neovascularization appears to be remarkably similar. The angiogenic process commences when endothelial cells in the vessel wall are exposed to angiogenic factors from the surrounding tissue (37). The endothelial cells respond by a series of characteristic morphological and functional changes in which the cells proliferate, enzymatically degrade the basement membrane, migrate toward the angiogenic stimulus, form and join capillary sprouts to create a capillary loop and a functional lumen, and finally deposit a new, initially fragmented and leaky basement membrane (38). Recently, putative endothelial cell
progenitors or angioblasts were found in human peripheral blood (39). In animal models, these endothelial progenitor cells were found to incorporate into sites of active angiogenesis.

While the actual angiogenic process is similar in normal and tumor tissue, there seem to be some generic differences in regulation of the angiogenic process, as well as both the architecture and function of the resulting vessels. In physiological situations, angiogenesis is turned off once the process is complete. In contrast, angiogenesis in cancer is not self-limiting - once tumor-induced angiogenesis is turned on it continues indefinitely (unless the tumor mass is dormant) until the tumor is eradicated or the host succumbs to the disease (40). Second, tumor angiogenesis results in a wide variety of structural and functional anomalies, including tortuosities, dilatations, arterial-venous shunts, and trifurcations which are not found in normal blood vessels (40). In addition, tumor-associated blood vessels have poorly developed basement membranes and lack collaterals, innervation, and pericytes which may lead to vessel leakiness, a common characteristic of tumor vessels. These abnormalities in blood vessel architecture result in disturbances in blood flow which may account for various features of tumor tissue such as necrosis and high interstitial pressure which is thought to have negative implications for drug delivery (40).

The angiogenic ‘switch’

In 1971, Folkman (4,5) proposed that solid tumor growth is angiogenesis dependent. As such, "once tumor take has occurred, every further increase in the tumor cell population must be preceded by an increase in new capillaries which converge upon the tumor" (38). Avascular tumors remain small, restricted in their growth by a poor supply of oxygen and nutrients which
are obtained only by diffusion (29).

Small tumors, unable to induce angiogenesis, stop expanding and reach a steady state, in which the number of dying cells counterbalances the number of proliferating cells (41). This restrains the increase in tumor volume that results from continuous proliferation, the hallmark of cancer. Thus, tumor masses that are not angiogenic are generally prevented from enlarging to a size that is clinically detectable and the cancer remains dormant (42). Tumor dormancy is defined as a population of tumor cells that is not increasing in size (42). Tumor dormancy results either because the cells are in cell-cycle arrest, or because there is a balance between proliferation and death in the population of tumor cells. In this regard, the interrelationship between tumor angiogenesis and cell death has been gaining attention. It has been shown that in certain tumors, necrotic cellular death follows from inadequate blood flow and hypoxia. For example, in glioblastoma, areas of necrosis have been attributed to hypoxia as a result of inadequate vascularization (43). In a study of gastric carcinoma, spontaneous apoptosis was found to be inversely related in intratumoral microvessel density (44) and it is now becoming clear that rapid expansion of a tumor mass can be significantly affected by the incidence of apoptosis. Folkman (45) demonstrated that tumor growth can be restricted by apoptosis of tumor cells, due to the inhibition of angiogenesis. When tumor bearing mice were treated with various inhibitors of the angiogenic process, tumor growth was impaired, concomitant with reduced vascularization and increased apoptosis, but without any change in cell proliferative index. Thus, it is clear that angiogenesis or lack thereof, plays a central role in the dormancy of certain tumors.

After months or years in steady state, a tumor may abruptly induce angiogenesis and be released from dormancy. The switch of a tumor from an avascular to a vascular phenotype, often
referred to as the 'angiogenic switch', occurs during the early stages of tumor development, suggesting that regulation of angiogenesis is a potentially rate limiting step in the pathway to many solid tumors (41,46). Solid tumor growth in vivo beyond 1-2 mm in diameter is, almost without exception, associated with the recruitment of new blood vessels (47,48). In melanoma, angiogenesis was found to commence when the lesion reached approximately 0.5-1mm in vertical thickness (29). Once the angiogenic process is turned on, the tumor mass may expand rapidly and begin to invade the surrounding tissue (41).

Experiments in transgenic mice bearing spontaneous tumors indicate that not all of the cells in a tumor switch to the angiogenic phenotype (45). Rather, only a small population of cells, approximately 4-10% become angiogenic. In human primary tumors, there are microscopic areas of intense neovascularization called 'hot spots' which are contiguous to areas of lesser neovascularization (41). This suggests that a tumor mass contains a heterogeneity of clones of highly angiogenic cells, as well as clones of tumor cells that are weakly or non-angiogenic. By recruiting new capillaries, the angiogenic cells may support the growth of both the angiogenic and non-angiogenic tumor cell populations (41).

Our laboratory (49) has proposed a model of tumor development which describes the relationship between the tumor cells and the endothelial cells as symbiotic in nature. In this model, tumor cells stimulate the endothelial cell population to proliferate and migrate, while the endothelial cells stimulate the growth of the tumor cell population by the production of various growth factors and cytokines. This 'paracrine effect' of neovascularization, coupled with the perfusion of nutrients and oxygen and waste removal, facilitates the expansion of a tumor or its metastases.
Mechanisms of the angiogenic switch

The switch to the angiogenic phenotype is thought to depend on a net balance between angiogenesis stimulators and inhibitors exported by the tumor cells, mobilized from the extracellular matrix, or released by infiltrating inflammatory cells such as macrophages (41). In some tissues, the relative absence of angiogenesis stimulators may keep the switch “off”, while in others, angiogenesis stimulators are present but held in check by relatively higher levels of angiogenesis inhibitors. As such, either reducing the overall concentration of inhibitors, or increasing the levels of stimulators, can change the balance and activate the switch leading to the growth of new blood vessels. Thus, the angiogenic switch is controlled by a regulatory mechanism in which the sum of the levels of stimulator and inhibitor signals either maintains the endothelial cells in either a state of quiescence or promotes angiogenesis (figure2).

Angiogenesis stimulators

The observation that tumors could be implanted in an avascular tissue, such as the cornea and thereafter stimulate the growth of new capillaries in the avascular tissue, suggested that tumors release diffusible angiogenesis stimulators. Indeed, inducers of angiogenesis have been identified using a number of in vitro and in vivo bioassays which have been developed which mimic various aspects of the angiogenic process.
The switch:

Activators
- aFGF
- bFGF
- VEGF
- ...

Inhibitors
- Thrombospondin-1
- 16 kD Prolactin
- Interferon α/β
- Platelet factor-4
- Angiostatin
- ...

Fig. 2 The balance hypothesis for the angiogenic switch (52).
In 1982, the first angiogenesis stimulator, namely basic fibroblastic growth factor (bFGF) was discovered, followed by the discovery of acidic fibroblastic growth factor (aFGF). Both proteins are members of a family of growth factors which are characterized by their high affinity binding to heparin and both have been found to be strongly chemotactic and mitogenic for endothelial cells (50). As discussed earlier, bFGF is expressed in human melanoma but not in melanocytes and has been shown to be an important autocrine factor for melanoma cells (51). On the other hand, aFGF is less frequently expressed in melanoma (51). Both acidic and basic FGF are unusual in that they lack the signal sequence which is necessary for protein secretion, yet under certain circumstances, they can be released from cells (52). Other members of the FGF family of proteins including keratinocyte growth factor (KGF) and FGF5 are expressed in both melanoma and normal melanocytes (51). Although some of these factors have been shown to have angiogenic activity, it is not yet certain whether these factors are involved in melanoma progression and angiogenesis (29). A recent publication by Becker et al. (53) demonstrated that liposome-mediated gene transfer of antisense-oriented bFGF and/or FGFR-1 cDNA resulted in regression or complete growth arrest of human melanoma tumors growing subcutaneously in nude mice. This effect was shown to be the result of blocked intratumoral angiogenesis and subsequent necrosis. Thus, bFGF and its receptor may be important in tumor formation in melanoma.

Another growth factor which has been implicated in angiogenesis is TGF-α. Secreted by transformed fibroblasts, macrophages and several tumor cell types, TGF-α has been shown to stimulate endothelial cell proliferation and angiogenesis (50). TGF-α has not been detected in normal melanocytes but its expression is induced in melanoma, suggesting a potential role in the
development of the disease (51). Another stimulator of angiogenesis, epidermal growth factor (EGF), shows a structural homology to TGF-α; however its angiogenic regulatory properties are less clear (54). Both EGF and its receptor have been shown to be present in melanomas (12,51).

Unrelated to TGF-α but bearing a similar name, the TGF-β family of proteins consists of at least three members TGF-β 1,2,3 (55). Paradoxically, although TGF-β shows an inhibitory effect on endothelial cell proliferation, it can induce angiogenesis in vivo (56). TGF-β1 and 3 are expressed in both normal and malignant melanocytes, whereas TGF-β2 is only expressed in melanoma cells (51).

Tumor necrosis factor alpha (TNF-α) is a cytokine which is produced mainly by activated macrophages in association with an immunological or inflammatory response (57). The effects of TNF-α on the angiogenic process are controversial; TNF-α has been shown to be inhibitory for endothelial cell proliferation in vitro, yet in vivo, TNF-α has been shown to induce neovascularization (58). These conflicting results have been attributed to a dose-dependent effect of TNF-α on angiogenesis. With respect to melanoma, TNF-α expression in human xenografts has not been detected (50).

In human cutaneous melanoma, interleukin-8 (IL-8) has been shown to be induced, in a dose dependent manner, by UV-B irradiation (59). In this study, UV-B irradiated melanoma cells displayed increased tumorigenicity and metastatic potential in nude mice which was attributed to the theory that IL-8 is a macrophage-derived mediator of angiogenesis. On this note when human recombinant IL-8 was implanted in the avascular rat cornea it was found to be potently angiogenic. In vitro, IL-8 was shown to induce proliferation and chemotaxis of human umbilical vein endothelial cells (HUVEC). Moreover, an IL-8 antisense oligonucleotide
specifically blocked the production of monocyte-induced angiogenic activity (60) demonstrating that IL-8 may induce angiogenesis in both a direct and indirect fashion. However, while IL-8 receptors may be present in endothelial cells \textit{in vitro}, they are not detectable in such cells \textit{in vivo} (61). This raises doubts about the role of IL-8 as a directly acting angiogenic growth factor.

\textbf{Vascular endothelial growth factor}

One of the most potent angiogenic factors is vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF). VEGF is a highly conserved, 34-42 kDa homodimeric glycoprotein, which shares homology with platelet derived growth factor (PDGF) (62). Initially identified by its ability to elicit vascular permeability, VEGF has also been shown to act as a survival factor for newly formed blood vessels (63). Consistent with its role as a potent angiogenic factor, receptors for VEGF were found to expressed on activated endothelial cells (64).

Recent experiments have demonstrated the pivotal importance of VEGF to the process of blood vessel formation. In 1996, it was reported by two groups that the loss of only a single VEGF allele is lethal in the mouse embryo (65). This lethality was found to be the result of abnormal blood vessel development in the VEGF knockout mice embryos.

The gene for human VEGF, located on chromosome fragment 6p21.3 (66) is organized into 8 exons. The promoter region of the gene has been shown to contain a single transcription start site and potential binding sites for Sp-1, AP-1 and AP-2 (67). As a result of alternative splicing, at least 4 transcripts encoding \( \text{VEGF}_{121}, \text{VEGF}_{165}, \text{VEGF}_{189}, \) and \( \text{VEGF}_{206} \) have been detected and seem to express identical biological activities. Yet these four isoforms do display
some differences. For example, VEGF$_{121}$ and VEGF$_{165}$ are readily diffusible, secreted proteins, whereas the two larger isoforms, VEGF$_{189}$ and VEGF$_{206}$ are found bound to heparin containing proteoglycans in the cell surface or in the basement membrane.

VEGF was originally purified on the basis of its affinity for heparin, yet the affinity of VEGF$_{165}$ is substantially lower than that of other typical heparin-binding growth factors such as bFGF, and the VEGF$_{121}$ isoform does not bind heparin (68). The heparin binding ability of VEGF$_{165}$ may be required under conditions in which oxidizing damage and free radicals are produced such as during hypoxia or inflammation (69). In these conditions, heparin may restore the activity of damaged VEGF$_{165}$. The relative insensitivity of VEGF$_{121}$ to oxidative damage may explain why this particular isoform does not require heparin for its activity (69).

Recently, two other related molecules, namely VEGF-B (70) and VEGF-C (71) have been uncovered. Both VEGF-B and VEGF-C are angiogenic molecules although their precise role in tumor angiogenesis is not yet known.

**VEGF receptors**

VEGF acts selectively on vascular endothelial cells through receptors on the cell surface, three of which have been identified and cloned. The VEGF receptors, flt-1 (fms-like tyrosine kinase) also known as VEGFR-1, and flk-1 or its human homologue KDR (kinase insert domain containing receptor) also known as VEGFR-2, are classified as Class III receptor tyrosine kinases. Both the flt-1 and KDR VEGF receptors are expressed predominantly in newly formed vascular endothelium. At sites of VEGF overexpression, both flt-1 and KDR are commonly and strikingly overexpressed in adjacent microvascular endothelial cells, although mutational analysis
implicates KDR, but not flt-1, in the VEGF induction of endothelial cell proliferation (72). KDR has also been reported to be expressed on certain hematopoietic cells such as stem cells, megakaryocytes, platelets, some human leukemia cell lines (73), and in certain melanoma cell lines (74). However, only endothelial cells have been reported to proliferate in response to VEGF. Interestingly, there is some selectivity in the binding of the various VEGF isoforms to their receptors. For example, it was shown that the VEGF_{121} isoform binds selectively to KDR (69). Recently, a third VEGF receptor, namely flt-4 or VEGFR-3, has been cloned. VEGFR-3 binds only VEGF-C and its expression becomes restricted mainly to the lymphatic endothelium during development (71).

Several effects follow the binding of VEGF to its receptor. Like other growth factor transmembrane tyrosine kinase receptors, VEGF receptors presumably undergo ligand-induced dimerization followed by aggregation of the receptor with proteins that contain SH2 domains and the subsequent activation of various signaling pathways (75). VEGF has been shown to activate both the phophotidylinositol 3-kinase (PI3-K) and the phospholipase C (PLC)-protein kinase C (PKC) signal transduction pathways (76). Cell signaling studies have shown that VEGF appears to mediate its effects partly through the PLC and PKC pathway and not the PI3-K pathway (76), which may be involved in the other consequences of VEGF binding to its receptor.

In response to VEGF, endothelial cells transiently accumulate cytoplasmic calcium, change their shape, undergo cell division and migration and altered gene expression (68). In a study comparing the signal transduction properties of KDR and flt-1, it was shown that KDR but not flt-1 transduced signals for mitogenicity, chemotaxis, actin reorganization, and changes in gross morphology despite the fact that flt-1 bound VEGF with higher affinity than KDR (77).
VEGF expression and biological activity

VEGF is a multifunctional cytokine which is expressed in many adult organs including the lungs, kidneys, adrenal gland, heart, liver, stomach mucosa, as well as in activated macrophages (78). The prominence of VEGF mRNA in these tissues suggests a potential role for VEGF in regulating baseline microvascular permeability important in tissue homeostasis. VEGF was originally discovered because of its ability to increase the permeability of microvessels to circulating macromolecules (68). VEGF increases microvascular permeability by enhancing the functional activity of a recently described organelle, the vesicular-vacuolar organelle (VVO) (79). In response to VEGF, these VVOs provide an 'open' fenestrated pathway through which plasma and plasma proteins may exit the circulation and enter the tissues. In normal adults tissues, extravasation of circulating macromolecules from the microvasculature is limited, but in tumor associated vessels VVO function is substantially upregulated (68). By rendering microvascular endothelial cells hyperpermeable, the microvasculature leaks plasma proteins into the extravascular space, which leads to the clotting of extravasated fibrinogen and the formation of a fibrin gel which serves as a provisional matrix that supports the growth of new blood vessels (68). In this way, VEGF facilitates angiogenesis in an indirect manner.

Aside from the baseline expression of VEGF in normal tissue, a high percentage of malignant animal and human tumors have been shown to overexpress VEGF. In tumors, VEGF has been shown to be concentrated in the tumor associated blood vessels (80). The importance of VEGF as a tumor angiogenesis factor was substantiated by demonstrating that VEGF expression is a prerequisite for tumor growth. For example, exogenous overexpression of VEGF in chinese hamster ovary (CHO) cells fails to provide the cells with a growth advantage in vitro,
yet they formed tumors \textit{in vivo}, unlike their non-transfected counterparts (81). Also, the transfection of cell lines representing various tumor types such as breast (82) and late stage melanoma (83) has been shown to increase angiogenesis, tumor growth and experimental metastases. Inhibition of VEGF function in tumors has also highlighted the importance of this molecule in development of metastatic disease. The \textit{in vivo} growth of glioma cell lines was substantially inhibited by the expression of an antisense VEGF sequence (84). Furthermore, injection of VEGF neutralizing antibodies inhibits the growth of primary tumors and suppresses the growth of metastases in mice, without affecting the proliferation of the tumor cells \textit{in vitro} (85,86). Similarly, interfering with VEGF receptor function in endothelial cells by expression of a dominant negative mutant form of the flk-1 VEGF receptor reduced the growth of a glioblastoma cell line in mice (87). These studies demonstrate that in many cases, inhibition of VEGF alone is sufficient to prevent tumor growth and dissemination \textit{in vivo}.

Aside from the fact that many tumors overexpress VEGF, the expression of VEGF can vary with tumor progression. For example, in brain, bladder, kidney, ovarian and gastrointestinal tract tumors, expression of VEGF increases with progression of the disease to greater malignancy and seems to correlate with the extent of tumor vascularization (88). Furthermore, these observations may have significant clinical implications. For instance, in primary breast carcinoma, VEGF expression was associated with high microvessel density and poor prognosis due to early relapse (89).
VEGF regulation

Recent studies have been undertaken to determine the mechanisms regulating VEGF expression. Since constitutive overexpression of VEGF has been associated with vascularized tumors growing in vivo, it is reasonable to suspect that various oncogenes and/or tumor suppressor genes may regulate VEGF expression. Our laboratory recently demonstrated that VEGF is substantially upregulated by intestinal epithelial cells overexpressing a mutant H-ras oncogene (90). We also showed that in colon carcinoma cells with the single mutated K-ras allele knocked out, VEGF production was significantly reduced (90). A similar observation was reported by another laboratory using H-ras transfected fibroblasts (91). Furthermore, it was reported that upregulation of VEGF correlates with the level of activated H-ras expression and the stage of progression of mouse skin carcinogenesis (92). Recent work by Folkman et al. (93) has shown that inhibition of phosphatidylinositol-3-kinase, (PI3K) which is activated by the ras oncogene, leads to partial but not complete inhibition of tumor angiogenesis. Thus, oncogenic H-ras may stimulate tumor angiogenesis through two distinct pathways, one of which involves PI3-K.

Aside from the ras oncogenes, the overexpression of various other oncogenes and tumor suppressor genes have been shown to alter the expression of VEGF. For instance, transient overexpression of v-src, was observed to upregulate VEGF in a dose dependent manner (94). Likewise, wild-type p53, but not mutant p53, was found to down-regulate endogenous VEGF mRNA levels in a dose-dependent manner (94). Another tumor suppressor gene, namely, the von Hippel-Lindau (VHL) gene has been shown to control VEGF expression. Human renal carcinoma cells that either lacked endogenous wild-type VHL, or were transfected with an
inactive mutant VHL, showed deregulated expression of VEGF and this was reverted by introduction of wild-type VHL (95). In this regard, the regulation of VEGF by VHL has been shown to occur at the post-transcriptional level (77).

Aside from changes in oncogenes and/or tumor suppressor genes, VEGF can also be regulated by changes in the tissue microenvironment. Deficient vascularization of malignant tumors results in the development of different microenvironments within the tumor mass that may in turn affect gene expression. For instance, perfusion insufficiency and resulting hypoxia often leads to neovascularization in order to satisfy the needs of the tissue (96) and there is sufficient evidence to suggest that local oxygen concentrations regulate VEGF expression. In tumor specimens, VEGF has been found to be specifically induced in areas in immediate proximity to hypoxic, necrotic foci (96). Moreover, in tumors with hypoxic foci, VEGF may be expressed not only by malignant cells but also by stromal cells (97). *In vitro*, VEGF levels are dramatically increased within a few hours of exposing different cell cultures to hypoxia and returns to background levels when the normal oxygen supply is resumed (96). Furthermore, when glioma tumor cells were grown as multicellular spheroids, the resulting hypoxia induced VEGF expression in the core of the spheroids (98).

It has been shown that hypoxia responsive enhancer elements are located at the 5' and 3' prime flanking regions of the human VEGF gene (99). Moreover, the VEGF sequence shares elements with another oxygen-sensitive gene, namely erythropoietin (99). It has been found in one study that hypoxic induction of human VEGF expression was found to occur through *c-src* activation (100). Expression of either a dominant negative form of *c-src* markedly reduced VEGF induction by hypoxia. Recently, it has been reported that oncogenic transformation and
hypoxia act synergistically to modulate VEGF expression (101). Mutant H-ras was shown to either constitutively or transiently enhance the induction of VEGF by hypoxia. However, although increases in the steady state levels of VEGF mRNA are partly due to transcriptional activation, they are mostly due to an increase in mRNA stability (102).

Acute glucose deprivation, another consequence of vascular insufficiency, also activates VEGF expression. In tumor spheroids, VEGF is upregulated in the areas of glucose deprivation (98). Similar to hypoxia, induction of VEGF expression by hypoglycemia is, for the most part, due to mRNA stabilization (102). These findings suggest that various stress factors in the local microenvironment may act to upregulate VEGF induced angiogenesis.

VEGF may also be regulated by various growth factors and cytokines. Unlike the effect of hypoxia, the effects of cytokine or growth factor stimulation of VEGF expression have been found to be target cell specific (103). For example, TGF-α and to a lesser extent EGF stimulate VEGF production in keratinocytes whereas other cytokines such as TGF-β, IL-1β and PDGF upregulate VEGF expression in a variety of other cell types. However, independent of cell type, VEGF overexpression by cytokine or growth factor stimulation is an indirect mechanism by which these molecules may promote angiogenesis. For example, IL-6 expression is elevated in tissues that undergo active angiogenesis but IL-6 does not induce proliferation of the endothelial cell itself (104). Furthermore, various cell lines tested were found to undergo significant induction of VEGF expression after treatment with IL-6 (104), thus promoting angiogenesis in an indirect fashion. The precise molecular mechanisms governing the expression of VEGF are not yet well characterized, and it is likely that other cytokines and growth factors may also promote the expression of VEGF.
Relevant to melanoma, UVB has also been shown to be a potent inducer of VEGF expression (105). In the latter study, it was observed that there was a large increase in VEGF mRNA and protein levels upon irradiation of quiescent keratinocytes with sublethal and physiologically relevant doses of UVB. Furthermore, UVB induced overexpression of VEGF was dependent upon de novo protein synthesis. This was speculated to occur via the release of soluble mediators which then turned on VEGF expression.

**Angiogenesis inhibitors**

Although an increase in the production of positive angiogenic factors is necessary for expression of the angiogenic phenotype, this alone is not sufficient. Most cells also produce inhibitors of angiogenesis which must be downregulated before the cells can develop into angiogenic tumors. More than fifteen natural inhibitors of angiogenesis have thus far been identified.

Of several naturally occurring angiogenesis inhibitors, thrombospondin was the first for which it was demonstrated that the inhibitor is produced constitutively by normal cells but downregulated during tumorigenesis (106). When tumor cells become angiogenic, they produce only 4-6% of the thrombospondin originally generated by their normal precursors. In human fibroblasts, thrombospondin is normally under control of the p53 tumor suppressor gene (107). In normal cells, p53 inhibits angiogenesis by stimulating the production of thrombospondin. However, fibroblasts from cancer prone patients with the Li-fraumani syndrome have only one copy of p53, and when this allele is either mutated or deleted, thrombospondin production is decreased and angiogenic activity is turned on.
Angiostatin (108), an internal fragment of plasminogen which is thought to be cleaved by metalloelastase (109), is a specific inhibitor of endothelial cell proliferation. Angiostatin was isolated from a subclone of a Lewis lung carcinoma in which the primary tumor inhibited the growth of its metastases, and was subsequently shown to be a potent inhibitor of angiogenesis. Furthermore, systemic therapy with angiostatin led to the maintenance of metastases in a microscopic dormant state. This was found to result from the balance between cell death due to apoptosis and proliferation with no net gain in tumor size, caused by the suppression of angiogenesis.

Recently, endostatin, a specific inhibitor of endothelial proliferation and a potent angiogenesis inhibitor, was isolated using similar rationale as for the isolation of angiostatin (110). Endostatin is the C terminal fragment of collagen XVIII. Systemic use of endostatin in anti-cancer therapy has led to the nearly complete suppression of tumor-induced angiogenesis, which resulted in a strong antitumor activity in tumor-bearing animals.

**Angiogenesis inhibitors as a strategy in the treatment of cancer**

In light of the problems associated with conventional chemotherapy, several features of the tumor associated vasculature make anti-angiogenic therapy an attractive anticancer therapeutic agent. With respect to melanoma, anti-angiogenic therapy may be particularly important because melanoma is relatively resistant to both chemotherapy and radiotherapy which are used primarily for palliative but not curative purposes (111).

First, unlike conventional chemotherapy, anti-angiogenic therapy generally has low toxicity. Because it is directed primarily at features specific to proliferating tumor-associated
capillary endothelial cells and not the normal resting endothelium, its side effects should theoretically be limited. Clinical trials have demonstrated that anti-angiogenic therapy does not cause bone marrow suppression, gastrointestinal symptoms, or alopecia which are often seen in cancer patients receiving conventional chemotherapy (41).

Second, drug resistance has not been found to be a significant problem in animal and clinical studies and proliferating endothelial cells have developed little or no resistance to angiogenesis inhibitors (41). This is because endothelial cells are genetically stable and, as a result, their capacity to develop drug resistance should be minimal. In contrast, tumor cells are genetically unstable and this feature allows them to develop acquired tumor cell resistance to therapeutic agents (112).

Third, the accessibility of endothelial cells to pharmacological agents in the blood makes them particularly attractive. Targeting the tumor vasculature is also a strategy that might circumvent the problem of limited penetration frequently encountered when delivering high molecular weight drugs to solid tumors with elevated interstitial pressures (40,113).

Fourth, the effect of vascular damage of tumor growth may be amplified by the fact that the viability of numerous tumor cells depends on a much smaller number of functional capillaries. There are approximately between 10 to 100 tumor cells for every endothelial cell in a tumor (114); therefore when an angiogenic inhibitor halts the growth of endothelial cells, the effect on the tumor cell population will be greatly amplified.

Finally, a combination of anti-angiogenic therapy and cytotoxic therapy can be curative in tumor bearing animals for which either agent alone is only partially inhibitory (115). Folkman (116) proposed that for therapeutic purposes it is useful to think about a tumor in terms of two
distinct cell populations or compartments, a tumor cell population and an endothelial cell population. Because each of these populations may stimulate growth of the other, combination treatment that selectively targets the tumor and endothelial cell populations i.e. cytotoxic chemotherapy and anti-angiogenic therapy, may have a synergistic effect, in vivo. It is conceivable that angiogenesis inhibitors may eventually be used to augment conventional chemotherapy.

Aside from the attractive elements of the use of anti-angiogenic therapy in cancer treatment, problems do exist with this type of approach. The central problem is to achieve selectivity by targeting the tumor associated endothelium and not the normal endothelium (40). However, even if complete selectivity cannot be achieved, this does not exclude anti-angiogenic therapy as a useful means to control cancer. An example of this is the widespread use of chemotherapy and radiation in the treatment of cancer, despite their obviously limited selectivity.

Another problem is that complete kill of tumor populations may not be possible because small tumors, i.e. those less than 1mm in diameter, can survive in the absence of a vasculature (40). On this note, anti-angiogenic therapy may be useful as a means to control tumor growth and stabilize the disease, as opposed to attempting to achieve complete tumor kill, as is the goal of conventional chemotherapy.

Background

The ability to address specific biological questions regarding melanoma development and progression has been facilitated because of the availability of cell lines obtained from different stages of melanoma progression. Human melanoma cell lines have been classified by Herlyn and
co-workers into three major classes or groups depending upon the clinical and histopathological features of the tumor that the cells were derived from (117). These three groups are comprised of the “early”, “intermediate”, and “advanced” melanomas. The early stage group were derived from either RGP or thin VGP primary human melanoma lesions. These patients have been followed up for 7-12 years in the absence of any sign of recurrence of the disease of metastasis after surgical removal of the primary tumor. The intermediate stage group is comprised of cell lines which were derived from primary tumors that can be defined as early stage by some parameters but as advanced stage by others. Such tumors seem to undergo a transition from early stage to advanced stage or they contain mixtures of tumor cells of both stages. Patients bearing an intermediately classified tumor had no evidence of metastases at the time of the removal of the primary tumor, but had metastases or second primary tumors at a later time. The advanced stage group refers to many human melanoma cell lines established from either thick VGP primary tumor in patients who had detectable metastatic disease at the time of diagnosis, or from metastatic lesions themselves.

From studies based upon such cell lines, as well as human specimens, it has been shown that in melanoma development, tumor progression is associated with an angiogenic response (figure3). RGP melanoma represents an avascular, curable disease (30). During tumor progression, melanoma cell begin to synthesize autocrine growth factors such as bFGF (51). As discussed earlier, these factors not only act as autocrine growth factors for the tumor cells but are also potent angiogenic factors and thus may simultaneously drive tumor cell proliferation as well as tumor neovascularization (53).
Fig. 3 Interrelationship between angiogenesis and the stage of progression in malignant melanoma. Transition from RGP to VGP is frequently associated with histological regression of the lesion which coincides with a 'switch' to the highly angiogenic state. In the presence of endothelial cells and inflammatory cells the more aggressive melanoma cell populations (black) may acquire a growth advantage over their non-metastatic counterparts (light grey: dark gray represents intermediate phenotypes). These effects may be mediated by IL-6 and other cytokines that can selectively inhibit the growth of early stage melanoma cells. This cascade of events may facilitate tumor cell dissemination, metastasis, a second angiogenic switch at the metastatic site. (40).
In VGP, thickness of the lesion begins to increase and this is associated with the rapid acceleration of tumor growth in the dermis and a worse prognosis. Stromal cells, including fibroblasts and endothelial cells present in the dermis, can secrete cytokines which are inhibitory for early stage melanoma. Frequent regression of the tumor infiltrates is often observed and this has also been associated with the onset of angiogenesis (118) (figure 3). Under such selection pressures, cellular variants resistant to inhibitory cytokines may undergo clonal expansion. The resulting resistant clonal cell populations may respond by inducing angiogenesis since many such cytokines, such as IL-6, are also stimulators of angiogenesis.

As thin VGP lesions become thicker, their demand for blood vessels increases. There are significant increases in vascularity as melanoma progresses from thin to thicker lesions, although the exact location of the angiogenic switch in melanoma progression is not yet known. Studies in vitro indicate that highly aggressive malignant melanoma cell lines express greater amounts of endothelial cell growth stimulatory factors than their less aggressive counterparts (37). These factors combined with pressure from the local microenvironment such as hypoxia and glucose deficiency as well as changes in oncogenes or tumor suppressor genes, may results in blood vessels growing towards and into the tumor. Expansion of the tumor mass is made possible not only because of perfusion of blood through the tumor, but also because of paracrine stimulation of tumor cells by numerous growth factors and matrix proteins that are produced by new capillary endothelium (49). Furthermore, the formation of vessels provides an escape route through which the melanoma cells may metastasize.
Rationale

The WM1341b cell line was derived from an early vertical growth phase human melanoma. Like other cell lines classified as early stage melanoma, the WM1341B cell line does not form tumors when injected into nude mice or forms very small slow growing tumors, thereby recapitulating its behavior in humans. The WM1341b cell line displays some characteristics of 'tumor dormancy' when injected into nude mice. After 23 days in vivo, the cell line remains in the skin as a small, flat nodule. An obvious question, given what is known about tumor progression, is what the biological basis for the dormant phenotype of the WM1341b cell line due to?

Tumorigenic variants of the non-tumorigenic WM1341b cell line, along with two other early stage melanoma cell lines, namely WM35 and WM793, have been derived by co-injecting the parental cell lines with matrigel (119), a basement membrane extract. Cell lines, designated WM1341bP2N3, WM35P2N3, or WM793P2N3 were derived after two in vivo passages with matrigel. The cell lines were thereafter capable of forming tumors in both the presence and in the absence of matrigel. The behavior of such tumors in vivo was reminiscent of the aggressive behavior of late stage melanoma.

A study by Bonfil et al. demonstrated that matrigel increased angiogenesis and this was one of the factors which contributed significantly to the tumorigenic promoting properties of matrigel (120). This prompted us to speculate that onset of angiogenesis was a factor that allowed the tumorigenic variants of WM1341B to overcome tumor dormancy in vivo. Immunohistochemical studies have shown that human melanoma specimens produce the potent angiogenic factor VEGF in vivo (Vivi Ann Florenes, personal communication). A study
examining VEGF in human melanoma cell lines xenografted to nude mice revealed that the cell lines with low metastatic potential were found to have a low expression, in comparison to highly metastatic cell lines which expressed large amounts of VEGF, \textit{in vitro}, but not \textit{in vivo} (121). However, this study reported that there was a change in the vascular architecture in the highly metastatic cell lines, indicating that the pattern or the level of VEGF expression is important for tumor angiogenesis in melanoma. In a study by Claffey et al. (83) a late stage melanoma cell line transfected with VEGF\textsubscript{165} formed large, well vascularized tumors with little necrosis, whereas the same cell line transfected with antisense VEGF\textsubscript{165} formed smaller tumors with extensive areas of necrosis. Similar results were observed in a poorly vascularized breast cancer cell line which was stably transfected with VEGF\textsubscript{121} (82). In addition, the growth of human fibrosarcoma \textit{in vivo} was almost completely inhibited by the administration of a neutralizing monoclonal VEGF\textsubscript{121} antibody (122).

\textbf{Hypothesis}

Sufficient evidence has thus far been presented to suggest that the 'angiogenic switch' is a critical step in the progression of melanoma from an avascular, curable disease to a highly angiogenic and aggressive tumor which often carries a much less favorable outcome for the patient. Exactly where the angiogenic switch becomes turned on in melanoma progression has yet to be elucidated, however in many other tumor types the switch to the angiogenic phenotype seems to be an early event. Thus, it is reasonable to suspect that lack of an angiogenic phenotype may be one major cause of the indolent behavior of early stage melanoma, \textit{in vivo}. The working hypothesis of this thesis is that a deficiency in angiogenesis is a cause of the dormant behavior
the early stage melanoma cell line WM1341B *in vivo* and this dormant phenotype may be terminated by the constitutive overexpression of a major angiogenic factor such as VEGF₁₂₁. To test this hypothesis, research described in the next chapter using the early stage melanoma cell line WM1341B was undertaken. Essentially I asked whether enforced upregulation of VEGF₁₂₁ in WM1341B cells would make them overtly tumorigenic in nude mice, i.e. would 'break' their normally dormant behavior. The next chapter will describe results from these studies. A final chapter will discuss the implications of the data and offer possible future directions for this project.
CHAPTER 2

TERMINATION OF THE DORMANT PHENOTYPE
OF EARLY STAGE PRIMARY HUMAN CUTANEOUS MELANOMA BY
OVEREXPRESSION OF
VASCULAR ENDOTHELIAL GROWTH FACTOR*

*(Note: Portions of this chapter have been submitted for publication)
Introduction

Like most other human cancers, the development of primary malignant cutaneous melanomas is usually preceded by a prolonged period of pre-malignant dormant cell growth (117,123,124). For example, it can take as long as a decade for the small plaque-like primary melanoma lesions of slow-growing superficial spreading melanoma, known as the ‘radial growth phase’ (RGP), to evolve into more rapidly growing, expansile vertical growth phase (VGP) primary tumors (124), the latter of which are potentially capable of distant metastatic spread. The molecular and cellular basis for this malignant / tumorigenic switch is unknown, but could obviously involve one or more of the following major changes: (i) acquisition of an uncontrolled melanoma cell proliferative phenotype, (ii) increased cell survival properties, i.e., a relative failure of melanoma cells to undergo apoptosis, and (iii) induction of an angiogenic phenotype. These possibilities are clearly not necessarily mutually exclusive (45).

Of considerable interest is the fact that the dormancy of early stage RGP or VGP human cutaneous primary melanomas can be studied in an in vivo context as a result of an interesting feature of cell lines established from such lesions. Whereas such cell lines readily grow in serum containing monolayer cell culture systems in a manner that is very similar to melanoma cell lines established from advanced stage VGP primary or distant metastatic lesions (117,125,126), the same cannot be said of their relative in vivo growth properties, when grown as xenografts in nude mice. Thus, whereas the vast majority of human malignant melanomas cell lines give rise to progressively growing solid tumors after subcutaneous or intradermal injection in nude mice, early stage melanoma cell lines fail to do so (119). Past experience in Dr. Kerbel’s as well as Dr. Herlyn’s laboratory have demonstrated that such cell lines are either completely non-tumorigenic
even after orthotopic (subdermal) injection – or may give rise to very slow growing tumors which attain a maximum size of only a few cubic millimetres in diameter (117,119).

The low grade or non-tumorigenic phenotype of early stage melanomas in nude mice – which mimics in a rather remarkable way their indolent behavior in patients – provides an excellent opportunity to investigate possible factors which may be responsible for their clinically dormant behavior, and why this property is eventually terminated. Studies in Dr. Kerbel’s laboratory have evaluated several methods of ‘converting’ early stage melanomas into expressing a frankly tumorigenic phenotype in nude mice including the ‘Matrigel assisted growth’ method (127). This method involves the co-inoculation of tumor cells with Matrigel, a reconstituted basement membrane extract (119,128). Matrigel, which is liquid when maintained in the cold, but gels at body temperature (119,128), possesses a remarkable ability to enhance the growth of tumor cells in nude mice, including human cell lines, or even fresh tumor tissue fragments, established from such tumors as lung, breast, and prostate cancer – all of which are normally difficult to grow in nude mice (128-131). Dr. Kerbel previously reported that three non-tumorigenic early stage melanoma cell lines, called WM-35, WM1341B, and WM-793, all gave rise to rapidly growing solid tumors in mice when the cells were co-inoculated with Matrigel into athymic nude mice (119).

The last observation suggested to us that the defect in tumorigenicity of these cell lines may be due, at least in part, to a failure to induce a sufficiently strong angiogenic response. This is because the \textit{in vivo} tumor-promoting effects of Matrigel have been attributed to promotion of an angiogenic response by the injected tumor cells at the site of injection (132). However, the evidence for this is still quite preliminary, and it is clearly possible – given the many molecular
constituents of Matrigel (133) – that additional or alternative mechanisms of tumor growth promotion are involved. I therefore decided to undertake an investigation to determine whether a more direct approach to promoting the angiogenic competence of early stage melanoma cells would result in their acquisition of an overt tumorigenic phenotype. To this end I screened a panel of early stage melanoma cell lines for ability to produce vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) in cell culture. VEGF/VPF is currently considered to be a major angiogenesis promoting factor for most types of cancer (68,134,135), which may include certain types of melanoma (136). There are four isoforms of VEGF/VPF of which the two shorter ones VEGF/VPF$_{121}$ and VEGF/VPF$_{165}$ – are secreted and known to induce angiogenesis in a variety of in vitro and in vivo model systems (68,137). I found one early-stage melanoma cell line (WM1341B) to be VEGF/VPF deficient, in that it does not produce VEGF/VPF constitutively and is only weakly capable of doing so under hypoxia-like conditions. The latter observation is critical since hypoxia can significantly upregulate VEGF/VPF (96,102,138); hence VEGF/VPF negative cells in culture may still be able to produce enough VEGF/VPF in vivo to induce angiogenesis (83,121). The WM1341B cell line was therefore used as a recipient cell population for a gene transfection protocol in which sublines expressing high levels of VEGF/VPF$_{121}$ were isolated and subsequently screened for tumor formation in nude mice.

The decision to focus on VEGF/VPF as an angiogenesis-inducing factor was based on several considerations. First, unlike many other putative angiogenesis growth factors, such as bFGF, it has no known autocrine growth promoting effects for carcinoma or melanoma cells (81,134,139). Such effects could seriously complicate the interpretation of the in vivo growth
experiments. Second, if it could be shown that constitutive VEGF/VPF expression in WM1341B cells does indeed result in significant tumorigenic growth properties, such variant sublines could be used as a novel in vivo screening tool for any drug or agent having putative anti-VEGF/VPF, or anti-VEGF/VPF receptor, function. Given the significance of VEGF/VPF dependent pathways for tumor angiogenesis in general, ramifications of such a screening system would extend beyond melanoma.

This study shows that high grade (malignant) progressively growing, tumorigenic variants of WM1341B cells can indeed by derived by transfection of the VEGF/VPF121 gene. To our knowledge this is the first demonstration of such a major change, or conversion, in tumorigenicity brought about by transfer of the VEGF/VPF gene into a recipient population of transformed cells. As such, the results support the hypothesis that defects in angiogenesis may be one of the key factors accounting for the dormant phenotype of early stage primary human cutaneous melanomas.

Materials and methods

Cell Lines and Culture Conditions

The human melanoma cell line, WM1341B was kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia). This cell line was originally established from a patient specimen diagnosed as early stage primary melanoma on the basis of histological analysis and clinical outcome (117,119). The tumorigenic variant of this cell line, WM1341B-P3N2, was derived by co-injection of WM1341B cells with Matrigel into nude mice as previously described (119). The MeWo cell line was established from a patient specimen that was diagnosed as late stage, lymph
node metastatic melanoma. All melanoma cell lines were maintained in RPMI 1640 medium (Gibco-BRL, Grande Island, NY) supplemented with 5% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT). Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics Corporation (San Diego, CA). HUVECs were cultured in MCDB131 medium supplemented with 10% heat inactivated FBS, 5 ng/ml human recombinant basic fibroblast growth factor (Upstate Biotechnology, Inc.), and 10 units/ml heparin (Gibco-BRL).

**Transfection and Selection**

The VEGF/VPF expression vector, described previously (82), contains a full length cDNA encoding the human VEGF121 isoform cloned into pcDNA1NEO backbone plasmid with a cytomegalovirus (CMV) promoter and a neomycin-Geneticin-sulfate (G418) resistance selection cassette. For control transfections, the same vector was used in which the VEGF/VPF insert was enzymatically removed. DNA (2 µg) was delivered to the cells by using Lipofectin reagent (Gibco-BRL) according to the supplier's instruction. The transfected cells were selected in their standard culture medium containing 400 µg/ml G418 (Gibco-BRL). Isolated colonies were cloned, expanded and periodically treated with 400 µg/ml G418.

**mRNA isolation and Northern Blot Analysis**

Total cellular mRNA was isolated from cultured cell lines by Trizol (Gibco-BRL) as per the manufacturer's instructions. 50 µg of total RNA was loaded in each lane and then resolved on a 1% agarose gel containing 6.6 M formaldehyde, transferred to a Zeta-Probe GT membrane (Bio-Rad), hybridized at 65°C with a $^{32}$P-labelled cDNA probe and autoradiographed. A 200 bp
fragment of the full length VEGF₁₂₁ cDNA was used as a probe. A probe derived from the cDNA for GAPDH was used as a control for RNA loading.

**Measurement of Human VEGF/VPF Protein Levels in Conditioned Medium (ELISA)**

A commercially available human VEGF/VPF ELISA kit (R&D Systems, Inc., Minneapolis, MN) was used to quantitate VEGF/VPF in conditioned medium from WM1341B, WM1341B-P3N2, transfected cell lines, and MeWo in normoxic and hypoxic tissue culture conditions. Briefly, 2 X 10⁵ cells were plated per well in 24 well plates overnight, and then the medium was replaced with fresh medium. Hypoxic conditions were mimicked by adding 100 μM cobalt chloride (CoCl₂) to the culture medium, as previously described (99). Conditioned medium was collected after 24 hours, cellular debris was removed by centrifugation and the medium was stored at -70°C until VEGF/VPF quantitation. Cell number was determined immediately after medium recovery using a coulter counter ZM (Coulter Electronics Ltd., Luton Beds, England).

**Cell Proliferation Assay**

5 X 10³ human melanoma cells or HUVECs were seeded onto each well of a 96-well tissue culture plate, grown overnight, and then incubated with either 5 or 50 ng/ml of recombinant human VEGF/VPF or 10 μg of monoclonal A.4.6.1 anti-VEGF neutralizing antibody for 48 hours. The properties of the A.4.6.1 antibody have been described elsewhere (81,85). As a control, 5 ng of human recombinant VEGF/VPF was added to HUVEC which is known to proliferate in response to VEGF. During the last 6 hours of incubation, the cells were
pulsed with 2 μCi/well of \(^{3}\text{H}\)thymidine (Amersham). The plates were then harvested, and the incorporated radioactivity was counted in a Betaplate (Pharmacia Biotech, Inc., Ste. Anne de Bellevue, Quebec, Canada) liquid scintillation counter.

**Western Blot Analysis**

Protein was extracted from cellular lysates and 30 μg of protein was loaded into each lane of a 7.5% polyacrylamide gel and electrophoretically separated at 120 V under reducing conditions. Proteins were electrotransferred from the gel onto a Immobilon-P membrane (Millipore Corp., Bedford, Mass.) overnight at 4°C using 10 V, and hybridized with a 1:100 dilution of the C-1158 antibody (Santa Cruz) reactive against the KDR VEGF/VPF receptor. HUVECs, which are known to express this receptor, were used as a positive control.

**Soft Agar Colony Formation Assay**

Six well tissue culture plates were prepared with 0.8% agarose in regular culture medium. Overlayed on this bottom layer was a mixture of 0.4% agarose in regular culture medium containing 6 X 10^3 cells. Plates were incubated for two weeks and the colonies counted.

**Tumorigenicity Assay**

Cells were cultured to near confluency, harvested by brief trypsin-EDTA (Gibco-BRL) treatment, washed in PBS, resuspended at the appropriate density, and injected orthotopically into the subdermal region of dorsolateral flank of 4-6 week old female athymic nude (nu/nu) mice as described previously (119). Mice were obtained from Harlan Sprague Dawley
(Indianapolis, IN) and were anesthetized with 60 mg/kg pentobarbital (Nembutal sodium, Abbot, Montreal, Canada). Typically, mice were injected with either $10^6$ cells/mouse/50 µl or $10^7$ cells/mouse/50 µl of WM1341B, WM1341P3N2, VEGF/VPF transfected WM1341B, or MeWo cells. Some of the mice were also injected intraperitoneally twice weekly with 200 µg/0.2 ml/mouse of the monoclonal A.4.6.1 anti-VEGF/VPF neutralizing antibody, as described previously (85). The resulting tumors were inspected twice weekly and measured by using a Vernier's caliper. Tumor volume ($\text{mm}^3$) was calculated by using the standard formula $a^2 \times b/2$ were "a" is the width and "b" is the length of the horizontal tumor perimeter. The experiments were terminated based upon ethical considerations, the mice were autopsied and tumor tissue was removed and processed for immunohistochemical analysis. The minimum of 4 tumors have been evaluated in each experiment. In some cases, two tumors were generated on contralateral flanks of a single mouse. No significant impact of such bilateral growth on tumor growth kinetics was observed compared to a protocol with single injection per mouse.

**Immunohistochemistry**

Tumors derived from WM1341B-P3N2 and transfected clones were fixed in 10% formalin, paraffin embedded, and sectioned. The HBM45 antibody (Biogenesis Ltd., New Fields, England) (140) which is specific for melanocytes was used to distinguish tumor cells from the surrounding stroma. The color reaction was developed by using an anti-rabbit secondary antibody, the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine tetrahydrochloride (DAB) (Pierce, Rockford, IL) as a chromagen to obtain brown colouration. The A-20 anti-VEGF polyclonal antibody (Santa Cruz) was used at a dilution
of 1:200 in combination with a proper secondary antibody from the Histostain-SP kit (Zymed Laboratories Inc., San Francisco, CA) and 3-amino-9-ethyl carbazole (AEC) chromogen to reveal the antigen as a red signal. The anti-Ki67 rabbit polyclonal antibody NCLki67p (Novocastra Laboratoires Ltd., Newcastle, UK) which detects proliferating cells, was used at a 1:1000 dilution. The color reaction was developed by using an anti-rabbit secondary antibody, the Vectastain ABC Kit and DAB as above. The commercially available TUNEL label kit (Boehringer Mannheim) which detects apoptotic cells, was used according to the manufacturers instructions. The colour reaction was obtained by using DAB as above.

Results

*Dormant Phenotype of Early Stage Melanoma Cell Line WM1341B in vivo.*

As expected, when cells of the early stage human melanoma cell line WM1341B are orthotopically injected into the subdermal tissue of nude mice (even using $10^7$ cells per injection), tumors failed to form. Mice were followed for up to ten months. Interestingly, a small, flat nodule remained in the skin of each mouse for several weeks after injection. In order to determine whether lack of tumor growth is due to elimination of tumor cells or their inability to grow in the skin, tissue containing the tumor inoculum was removed at various time points, fixed, sectioned and stained with haemotoxylin-eosin or HBM45 antibody for the presence of viable human melanoma cells. As shown in Figure 4, clusters of viable WM1341B cells were detectable in the subdermal connective tissue up to 23 days after injection. Necrotic areas and inflammatory infiltrations were also present suggesting that in the absence of growth promoting stimuli, tumor cells may eventually be eliminated after an unusually long presence at the
Fig. 4 Immunohistochemical evaluation of WM1341B tumor dormancy in nude mice. WM1341B innoculates were examined 6 days (A, B) and 23 days (C, D) post injection. Tissue sections were stained with haematoxylin-eosin (A, C) and with the HBM45 antibody (B, D) which is reactive against human melanocytes. Viable melanoma cells were present both 6 and 23 days post injection, indicative of tumor dormancy. Human melanoma cells are shown as having a brown nuclear staining. (Bar=100 μm).
injection site. Since this behavior was reminiscent of what has been observed in the case of dormant, angiogenically deficient micrometastases (45), it was logical to suspect that these results could have a similar basis. Because angiogenesis in many tumor types is driven by expression of VEGF/VPF and a postdoctoral fellow in Dr. Kerbel’s laboratory found that this growth factor was readily detectable in human melanoma specimens by both immunocytochemical and mRNA analysis (Florenes et al. unpublished observations), I decided to assess VEGF/VPF production by WM1341B cells in tissue culture. By Northern analysis (figure 5) only, an extremely weak VEGF/VPF band could barely be observed, and secretion of VEGF/VPF protein into the conditioned medium was not detectable by a specific ELISA assay (Table 1).

**Overexpression of exogenous VEGF_{121} in Early Stage WM1341B Human Melanoma Cells**

In order to determine if a deficient ability to induce angiogenesis was a factor responsible for the *in vivo* dormant phenotype of the WM1341B melanoma, the cells were stably transfected with the cDNA encoding the human VEGF_{121} isoform under the control of a CMV promoter. A total of 25 G418 resistant colonies were isolated, cloned, expanded and screened for VEGF_{121} expression. The VEGF_{121} expressing clones were identified by a unique 1.9 kb mRNA band on Northern blots (Figure 5). Eleven out of 25 transfected WM1341B clones expressed exogenous human VEGF/VPF mRNA with endogenous mRNA (4.2 and 3.7 kb) barely detectable (Figure 5). The conditioned medium of all successfully transfected clones and control clones was then assayed for VEGF/VPF immunoreactivity by using a VEGF/VPF specific ELISA. While the parental cell line, WM1341B, did not express detectable levels of VEGF/VPF...
**Table 1.** In vitro and in vivo characterization of WM1341B, VEGF₁₂₁ transfected clones, WM1341B-P3N2, and MeWo.
Fig. 5 Northern blot analysis of VEGF/VPF mRNA in WM1341B cells which were transfected with a VEGF_{121} cDNA. VC stands for VEGF/VPF control (control clone containing VEGF_{121} insert). V1, V2, etc. stand for individual VEGF/VPF_{121} transfected clones.
protein, all but one of the successfully transfected clones expressed this protein, the relative levels of which were in reasonable agreement with their respective levels of exogenous VEGF/VPF mRNA (Table 1).

Overexpression of VEGF/VPF is sufficient to confer tumorigenic phenotype on early stage melanoma cells.

The transfected cell lines which produced the highest quantities of VEGF/VPF, as determined by ELISA (Table 1), namely V2, V22, V24, and V25, as well as their parental WM1341B counterparts, were first tested for their ability to grow in soft agar in order to assess their transformation status. All of the cell lines tested developed similar numbers of colonies (Table 1), thus suggesting that transfection or VEGF/VPF expression did not alter their intrinsic growth requirements and survival properties (data not shown). This would be expected if the cells lacked functional VEGF/VPF receptors, which is the case (see below).

While injection of parental WM1341B cells and control transfectants repeatedly failed to give rise to tumor growth in vivo (Table 1), in the case of VEGF/VPF transfected clones, tumor take became apparent within one week after injection of the cells into the subdermal connective tissue of nude mice. By forty days after injection, in 13 out of 16 mice injected with $10^7$ VEGF/VPF expressing cells (Table 1), and in 4 out of 16 mice injected with $10^6$ (data not shown) of such cells aggressively growing tumors were formed. These tumors had an apparent vascular network and red colouration (Figure 6A). Furthermore, the aggressiveness of tumor growth of the individual cell lines was in good agreement with their relative VEGF/VPF production level as detected by ELISA assay such that the cell lines could be aligned in the following order of
Fig. 6 The effect of transfection of VEGF121 on growth of WM1341B cells *in vivo*. A. Growth of WM1341B parental and various transfected clones up to 40 days post injection. B-D. Immunohistochemical evaluation of VEGF/VPF expression in transfected WM1341B clones V22, V24, V25 (B, C, D, respectively). VEGF/VPF expression is shown as red cytoplasmatic staining. (Bar=100m).
increasing tumorigenicity: V2>V25>V22>V24. All the tumor tissue sections derived from the transfected clones were positive for VEGF/VPF protein and contained a dense network of blood vessel capillaries as shown by immunohistochemistry (Figure 6B-D).

In order to further ascertain that tumor growth in the case of VEGF/VPF transfectants was indeed dependent on VEGF/VPF, mice injected with $10^7$ V2 and V25 cells were treated twice weekly, with the monoclonal A.4.6.1 anti-VEGF neutralizing antibody. The mice treated with A.4.6.1 either failed to develop tumors or developed much smaller tumors relative to the control group which did not receive antibody treatment (Figure 7). Interestingly, 75% of tumor-bearing mice injected with VEGF/VPF transfected cells developed symptoms of severe cachexia with loss of up to one-third of their body weight. These symptoms were suppressed by treatment with anti-VEGF/VPF A4.6.1 antibody and to a similar extent by surgical removal of VEGF/VPF producing tumors. In the latter case the recovery occurred within 2 weeks after surgery (data not shown).

In order to test whether growth in vivo of spontaneously derived tumorigenic melanoma cells is also dependent on VEGF/VPF production, two cell lines were selected. WM1341B-P3N2, is a tumorigenic variant of WM1341B cells which had been obtained after several rounds of co-injection of the latter cells with Matrigel (119). MeWo is a highly malignant melanoma cell line derived from lymph node metastasis of advanced human melanoma (141,142). Both cell lines were injected into nude mice and treated as described earlier with the A.4.6.1 antibody. Similar to the VEGF/VPF transfectants, an anti-tumor effect of antibody treatment was also observed in these cases, albeit to a somewhat lesser extent (Figures 8-9). Cachexia also developed in mice bearing WM1341P3N2 tumors and was alleviated by antibody treatment while
Fig. 7 The effect of treatment with A.4.6.1 monoclonal neutralizing antibody on growth of V2 (VEGF-transfected WM1341B clone) xenografts in nude mice. Mice were treated twice weekly with 200 μg of the A.4.6.1 antibody. Arrows represent treatment timepoints.
Fig. 8 The effect of treatment with A.4.6.1 monoclonal neutralizing antibody on growth of WM1341B-P3N2 (tumorigenic variant of WM1341B) xenografts in nude mice. Mice were treated twice weekly with 200 μg of the A.4.6.1 antibody. Arrows represent treatment timepoints.
Fig. 9 The effect of treatment with A.4.6.1 monoclonal neutralizing antibody on growth of MeWo (human metastatic melanoma) xenografts in nude mice. Mice were treated twice weekly with 200 μg of the A.4.6.1 antibody. Arrows represent treatment timepoints.
no such symptoms were observed in mice bearing MeWo tumors of comparable size.

*VEGF/VPF expression does not exert an autocrine effect on early stage melanoma cells in vitro.*

Since VEGF/VPF has been reported to act on various VEGF/VPF receptor positive non-endothelial cell types, I decided to examine whether overexpression of this growth factor in WM1341B cells can alter their growth properties in an autocrine manner. This is especially critical since it has been reported that a small minority of established human melanoma cell lines can express VEGF/VPF receptors such as KDR/VEGFR2 (74). I found that in both the WM1341B and the tumorigenic variant WM1341B-P3N2, the addition of 5 or 50 ng/ml of recombinant VEGF/VPF failed to induce any increase in proliferation in the standard *in vitro* [³H] thymidine incorporation assay (Figure 10). On the other hand, similar treatment had an expected dose dependent growth/survival promoting effect on human umbilical vein endothelial cells. Moreover, no change in thymidine incorporation of WM1341B, WM1341B-P3N2, and VEGF/VPF expressing clones was observed in cells treated *in vitro* with 5 ng/ml of the A.4.6.1 monoclonal neutralizing anti-VEGF/VPF antibody at the concentration sufficient to neutralize 80% of the growth promoting activity of 5 ng/ml of recombinant VEGF/VPF against HUVECs (Figure 11). Furthermore, I found that the parental WM1341B cell line, the tumorigenic variant WM1341B-P3N2, the transfected clones (V2, V24, and V25), did not express the VEGF/VPF receptor KDR protein, as detected by standard Western blot analysis (Figure 12). In addition both VEGF/VPF expressing and non-expressing clones of the WM1341B cell line had similar *in vitro* growth kinetics in either standard or serum free medium (data not shown) and they did not differ in terms of their colony forming efficiency in soft agar. Based on these results, I conclude
Fig. 10 Unresponsiveness of WM1341B cell line to exogenous VEGF/VPF in vitro. WM1341B and WM1341B-P3N2 cell lines do not alter their proliferative capacity in response to exogenously added human recombinant VEGF/VPF as assessed by thymidine incorporation.
Fig. 11 Unresponsiveness of WM1341B cell line to exogenous VEGF/VPF in vitro. Treatment of WM1341B, WM1341B-P3N2 and VEGF<sub>121</sub> transfected clones of WM1341B with 50 μg/ml of the monoclonal A.4.6.1 anti-VEGF neutralizing antibody does not affect their proliferative capacity as assessed by thymidine incorporation, in contrast, addition of A.4.6.1 antibody inhibits growth/survival of HUVECs pre-treated with 5 ng/ml of hrVEGF<sub>165</sub>. 
Fig. 12 The WM1341B, WM1341B-P3N2, and the VEGF/VPF121 transfected clones V2, V22, V24, V25 do not express the VEGF receptor KDR as assessed by western blot analysis in. Unspecific hybridization band and amidoblack staining were used as a loading control.
that the tumor promoting properties of VEGF_{121} expression can be attributed to stimulation of angiogenesis rather than to a direct autocrine effect on melanoma cells.

**The impact of VEGF/VPF over-expression on survival of melanoma cells in vivo**

It is implicit that the tumor growth promoting effect of VEGF/VPF expression and resulting angiogenesis would ultimately be executed through a change in proliferative and/or survival status of tumor cells themselves. To examine this question more closely, tissue sections obtained from the dormant inoculum of parental WM1341B cells (6 days post injection), WM1341B-P3N2 tumors, and from tumors generated by injection of VEGF/VPF overexpressing clones V2, V22 and V25 were analyzed for cell proliferation or cell death by staining with Ki67 antibody and TUNEL reagent, respectively. I was unable to reliably assess the rate of apoptotic cell death by TUNEL staining since no such specific staining could be detected even though apoptotic cells were readily detectable in the positive control sections of murine intestine and in various other tumor specimens. However, I have taken advantage of the non-specific staining of necrotic areas to qualitatively evaluate the extent of necrotic cell death and proliferation. This analysis revealed striking differences in the extent of necrosis between different tumor cell lines. Both the inoculum of parental WM1341B cells and the WM1341B-P3N2 tumor both had appreciable necrotic cores (figure 13 A), in contrast, the VEGF_{121} transfected clones displayed much less necrosis (figure 13 B-D). On the other hand, there was no appreciable difference observed between the inoculum of the various cell lines in terms of the extent of proliferation (figure 13). It can be concluded that in the case of VEGF/VPF transfectants, a highly expressed level of this protein can act as a survival factor for tumor cells *in vivo*. This seems to be the main
to stimulation of angiogenesis rather than to a direct autocrine effect on melanoma cells.

The impact of VEGF/VPF over-expression on survival of melanoma cells in vivo

It is implicit that the tumor growth promoting effect of VEGF/VPF expression and resulting angiogenesis would ultimately be executed through a change in proliferative and/or survival status of tumor cells themselves. To examine this question more closely, tissue sections obtained from the dormant inoculum of parental WM1341B cells (6 days post injection), WM1341B-P3N2 tumors, and from tumors generated by injection of VEGF/VPF overexpressing clones V2, V22 and V25 were analyzed for cell proliferation or cell death by staining with Ki67 antibody and TUNEL reagent, respectively. I was unable to reliably assess the rate of apoptotic cell death by TUNEL staining since no such specific staining could be detected even though apoptotic cells were readily detectable in the positive control sections of murine intestine and in various other tumor specimens. However, I have taken advantage of the non-specific staining of necrotic areas to qualitatively evaluate the extent of necrotic cell death and proliferation. This analysis revealed striking differences in the extent of necrosis between different tumor cell lines. Both the inoculum of parental WM1341B cells and the WM1341B-P3N2 tumor both had appreciable necrotic cores (figure 13 A), in contrast, the VEGF12i transfected clones displayed much less necrosis (figure 13 B-D). On the other hand, there was no appreciable difference observed between the inoculum of the various cell lines in terms of the extent of proliferation (figure 13). It can be concluded that in the case of VEGF/VPF transfectants high expressive level of this protein can act as a survival factor for tumor cells in vivo. This seems to be the main mechanism responsible for tumorigenic conversion in this case while other possibly intrinsic
Fig. 13 Staining of tumor sections for expression of Ki67 proliferation associated protein. (A), WM1341B-P3N2; (B), V2; (C), V22, and, (D), V25. Ki67 positivity appears as brown staining of the nuclei. Arrows indicate areas of necrosis.
mechanism responsible for tumorigenic conversion in this case while other possibly intrinsic cellular properties must contribute to tumor formation by WM1341B-P3N2 cell line.

The relationship between constitutive and inducible expression of VEGF/VPF during melanoma progression.

When constitutive production of VEGF/VPF protein was compared between WM1341B, WM1341B-P3N2, and MeWo cells the results were somewhat paradoxical. Thus no detectable VEGF/VPF was present in conditioned medium of WM1341B and WM1341B-P3N2 cell lines whereas MeWo cells were reproducibly positive (Figure 14), which was hardly in line with the tumorigenic properties of the respective melanoma cell lines. Since hypoxia is one of the strongest physiological inducers of VEGF/VPF in solid tumors (96), I decided to compare VEGF/VPF production of the various melanoma cells upon treatment with 100μM cobalt chloride which biochemically mimics an oxygen deficit (99). Under such conditions VEGF/VPF production was induced in both WM1341B and WM1341B-P3N2 cell lines, albeit the levels being approximately 3-fold higher in the latter case (figure 14). Since hypoxia alters not only VEGF/VPF transcription but also mRNA stability (96,99,138) combination of high constitutive VEGF/VPF expression and cobalt chloride treatment resulted in very high levels of VEGF/VPF production in the case of VEGF/VPF121 transfected clones of WM1341B (figure 14). Significant, up to six fold, upregulation of VEGF/VPF expression was also observed in MeWo cells exposed to hypoxia like conditions (figure 14). Taken together, these results suggest that melanoma progression from non-tumorigenic to a tumorigenic phenotype may not affect significantly the constitutive production of VEGF/VPF but rather its intrinsic inducibility by microenvironmental conditions.
Fig. 14 Differential upregulation of VEGF/VPF under hypoxia-like conditions in human melanoma cell lines. Secretion of VEGF/VPF into condition medium by control or CoCl₂ (100µm) treated WM1341B, WM1341B-P3N2, V2, V25 (transfected clones), and MeWo cells as assessed by ELISA.

Summary
The major finding of this study is that upregulation of VEGF/VPF – in this case of the VEGF/VPF121 isoform – is sufficient to endow early stage non-tumorigenic cutaneous melanoma cells with high grade tumorigenic competence in nude mice. To my knowledge, there is no published precedent for this finding. Although several groups have undertaken VEGF/VPF gene transfection studies using tumor cell lines as recipients – including human melanoma – the cell lines were highly tumorigenic to begin with (83,121,143). As a result of the transfection protocols and upregulation of VEGF/VPF, sublines were obtained which expressed either an enhanced tumorigenic or metastatic capacity in nude mice (83,143). In one study the starting cell line, like ours, was non-tumorigenic, namely VEGF/VPF negative, immortalized Chinese hamster ovary (CHO) cells (81). Transfection of a VEGF/VPF cDNA into such cells did result in a detectable growth advantage in vivo, but not in cell culture (81). However, the growth advantage was extremely modest: only very small benign tumors which stopped growing after a few weeks, formed at the site of inoculation (81). In contrast, I found that progressively growing large and essentially malignant tumors of the VEGF/VPF121 transfectant clones were able to form in nude mice.

These results are therefore highly suggestive of the possibility that the major defect in tumorigenicity of the early stage WM1341B melanoma cell population is due to an insufficient ability to produce angiogenesis stimulatory activities such as VEGF/VPF. In the following chapter I will discuss below several aspects of the results and their possible implications for melanoma pathobiology and tumor angiogenesis.
CHAPTER 3

DISCUSSION AND CONCLUSIONS
Although human cutaneous melanoma has been well characterized in terms of histological features and clinical diagnosis, there is clearly a gap in our knowledge which is inhibiting the ability of oncologists to develop effective therapies for late stages of this disease. That is, we have yet to uncover the precise factors are which drive the progression of melanoma from early curable stages to advanced incurable, highly malignant disease. Hopefully, as we gather more information regarding the genetic, molecular, and microenvironmental influences which drive melanoma progression, we will be in a better position to be able to develop agents which target these factors to slow, halt or reverse progression of the disease, before it enters a malignant state.

Cell lines derived from either early or late stages of melanoma show no obvious differences in growth kinetics or survival in monolayer culture in vitro, yet in vivo the growth of cell lines derived from these two different stages of the disease, is strikingly different. Early stage human melanoma cell lines fail to form tumors upon injection into nude mice whereas their later stage counterparts are almost always capable of doing so. Because tumor vascularity is a factor which can only be realized in vivo, it is conceivable that a deficiency in the ability to form a vasculature is inhibiting the early stage cell lines from forming tumors in nude mice. Temporally, the onset of tumor vascularization has been shown to correlate with melanoma progression from the early to late stage (30). Although important, the prognostic value of this observation is questionable. As reviewed in the introduction, some laboratories, including our own, have reported that relative blood vessel counts can be used to predict patient outcome, however other laboratories have reported the contrary. Clearly more work needs to be done in this area. Larger sample sizes and consistent vessel quantification techniques should be
employed in future studies of this kind.

Although it is not yet clear whether or not blood vessel counts in melanoma are a useful prognostic indicator, the onset of angiogenesis is obviously important from a biological and therapeutic perspective. Gene transfer studies in which a potent angiogenic factor encoding gene such as VEGF was transfected into melanoma cell lines, have shown that enhanced angiogenesis can increase tumorigenicity in human melanoma. It is important to note that several authors have reported an increase in tumorigenicity of cell lines which were known to be tumorigenic prior to transfection and selection. Furthermore, Ferrara et al. showed that transfection of a VEGF cDNA into CHO cells was sufficient to induce tumor formation, although such tumors were benign and very small in size (81), whereas no such growth advantage was observed in vitro. However, the question as to whether angiogenesis can drive tumor progression from an early stage of melanoma in which cell lines derived from such stages fail to form tumors in nude mice, to a later stage of the disease, i.e. where the cells would be frankly tumorigenic in nude mice, was not addressed until this study on melanoma was undertaken. Thus, this study was initiated to determine if the transfer of a gene encoding a factor which promoted angiogenesis into early stage curable melanoma would be sufficient to allow the cells to progress to an advanced stage. Indeed, as discussed in the previous chapter, transfection with VEGF_{121} conferred a tumorigenic phenotype upon an early stage melanoma cell line. The conclusions, potential implications, and future directions of this study are discussed below.
Overexpression of VEGF<sub>121</sub> is necessary but probably not sufficient to overcome tumor dormancy in the WM1341B human melanoma cell line.

In keeping with our *in vivo* model of melanoma progression, the early stage melanoma cell line WM1341B failed to form tumors upon injection into nude mice. However, I did observe aspects of tumor dormancy when the cells were injected into nude mice. It may be assumed, based upon the current definition of tumor dormancy, that the WM1341B cells were either growth arrested or were proliferating and undergoing cell death at a balanced rate in which there is no net change in size. In this study, the small mass of cells was excised from the mouse periodically during a period of 23 days. It would be interesting to continue this type of study for longer periods of time to monitor any changes that may prevail i.e. either growth or eventual regression of the dormant tumor mass.

For several reasons, it is logical to suppose that one of the major factors accounting for the dormant behavior of early stage primary melanomas is lack of angiogenesis. First, the transition from a curable RGP or thin VGP tumor to, for the most part, incurable, thick VGP lesions is always associated with, and likely requires an increase in vertical tumor diameter, the extent of which would exceed the experimentally predicted threshold (1-2 mm) of avascular tumor growth (30). Second, RGP or thin VGP primary lesions are generally confined to the epithelial epidermis where blood vessel capillaries are lacking (124). It is only as the lesions penetrate into the underlying dermis, where blood vessels are located, that the process of angiogenesis can begin in earnest. Hence, generation of new blood vessels can be considered a probable and logical pre-requisite for the RGP to VGP transition (30). It is important to keep in mind that melanomas can remain as slow growing thin RGP tumors for many years despite the
fact that upon histological examination, the tumor cells have a clearly transformed melanocytic appearance. This is why such primary melanoma cannot be considered a truly "pre-malignant" lesion but rather one resembling a malignant primary tumor in a growth dormant state.

As mentioned earlier, there are also correlative data suggesting that cessation of the early-stage primary melanoma dormancy may be associated with an increase in angiogenesis. For example, high blood vessel counts have been noted in some studies associated with poor prognosis in thin (28) and intermediate thickness (31) primary melanoma. However, the absence of such a correlation has also been reported (144). This is perhaps not surprising since intrinsic properties of melanoma cells may be as or more relevant for the frequency of metastasis than simple numerical changes in blood vessel counts. Furthermore, with the selection of increasingly malignant melanoma cell variants, the relative dependence of tumor cells on high blood vessel density may actually decrease (145). Interestingly, the high vascularity sometimes observed in thin melanomas has been found to be associated with evidence of histological regression, and this feature almost invariably predicts a more aggressive course of the disease (28,118). The latter, somewhat paradoxical, finding may actually be quite revealing since the regression could represent a mechanism for accelerated selection of highly malignant tumor cell populations (40). Dr. Kerbel’s laboratory has previously hypothesized that invading activated endothelial cells themselves may cause regression of previously avascular melanoma lesions through the secretion of paracrine growth inhibitory cytokines, such as IL-6 (49).

To create early stage melanoma cell lines which can induce angiogenesis in vivo, it seemed logical to transflect such cell lines with a VEGF gene. VEGF was the angiogenic factor of choice for a number of reasons. First and foremost, VEGF has been shown to be a specific
endothelial growth factor. In this regard, transfection with VEGF would serve only to induce angiogenesis and not have any side effects such as autocrine growth, as it is not known to be an autocrine growth factor for melanoma cells. Some angiogenic factors, such as bFGF (50), also behave as autocrine growth factors for melanoma and therefore would not be practical to use because it would be impossible to exclude the autocrine effects of such an angiogenic factor on growth in vivo. Second, based upon the observation that in normal monolayer culture, the WM1341B cell line fails to produce VEGF protein and only moderately upregulates VEGF under inducible hypoxic conditions, one can speculate that it is a lack of VEGF that is keeping the cells in this dormant state. Moreover, studies by Cavanee et al. (139), Wilks et al. (84) and our laboratory (Futoshi Okada, unpublished observations) have shown that 3-4 fold reductions in VEGF expression induced by antisense methods can result in profound inhibition of tumorigenicity of various human tumor cell lines in nude mice. Thus, VEGF was a logical angiogenic factor with which to transfect the WM1341B cell line.

Indeed, transfection of the WM1341B cell line with VEGF121 transformed the cells by in vivo but not in vitro criteria. When transfected cell lines, which differed presumably from the parental cell line only in that they constitutively overexpressed VEGF121, were injected into nude mice, tumor take occurred in almost every case. Furthermore, when mice injected with transfected cell lines were treated with the A.4.6.1 monoclonal anti-VEGF neutralizing antibody, there was a significant decrease in tumor growth and size. Thus, by simply neutralizing the VEGF protein I could, in effect, almost reverse the observed tumorigenicity associated with VEGF transfection. Cell lines derived from late stage metastatic melanoma or matrigel derived tumorigenic variants of early stage melanoma were also treated in vivo with the A.4.6.1 antibody.
In these cases tumor reduction was observed, although not as great as the tumor reduction observed with the VEGF transfected WM1341B cell lines. Thus, it may be inferred that late stage melanoma is less dependent upon VEGF than early stage cell lines. Late stage melanoma likely has many genetic alterations which confer growth advantages in vivo upon them, so that they may override the effects of neutralizing the VEGF by antibody administration. Furthermore, these cell lines may also upregulate a plethora of other angiogenic factors such as bFGF, IL-8, pleiotrophin, HGF and TGF-α (146-148) so that they may be less dependent upon VEGF for the promotion of angiogenesis. In our laboratory, late stage metastatic melanoma cell lines are currently being transfected with a VEGF antisense cDNA. It will be interesting to observe any changes in tumor growth which accompany this down regulation of VEGF. This study should answer some questions as to the dependence of advanced stage melanoma on VEGF.

From experiments involving either the A.4.6.1 antibody or the human recombinant VEGF protein, it was clear that it was angiogenesis itself, and not an autocrine effect of VEGF on the melanoma cells, which allowed the WM1341B cell line to overcome tumor dormancy in vivo. As a small number of human melanoma cell lines have been shown to express the VEGF receptor KDR (74), it was necessary to screen both the parental and transfected cell lines for KDR protein positivity and proliferation differences in the presence of VEGF.

The results of this study appear to implicate VEGF as a contributing factor in the progression of melanoma. However, until more definitive evidence becomes available on the relevance of VEGF to melanoma progression, the translation of such results towards greater clinical understanding should be taken with caution. Indeed, at this point there is no firm clinical evidence to suggest that VEGF expression is relevant to melanoma progression. Tumor sections
taken from human specimens have shown to express VEGF protein but there is no relationship between *in vivo* expression of VEGF protein and stage of disease (Vivi AnnFlorentes, personal communication). What this study does suggest though, in the context of the previously cited literature, is that angiogenesis, driven by the upregulation by some angiogenic factor(s), contributes to melanoma progression.

The question of whether angiogenesis alone is sufficient to allow cells to progress from the early to late VGP stage of melanoma remains unanswered. Cell lines, such as WM1341B, which have been derived from early VGP, may already possess other necessary genetic alterations which collaborate with angiogenesis to drive tumor progression. Furthermore, this study represents the transfection of a single cell line derived from early stage melanoma and therefore may or may not be representative of the behavior other early stage melanoma cell lines. More conclusive evidence for the involvement of angiogenesis in the RGP-VGP transition would require transfection of other melanoma cell lines derived from the early stage. Since cell lines from such stages are not only hard to come by but are also difficult to transfect, this represents a challenging but necessary task to future investigations.

It was also interesting to note that the tumorigenic variants of WM1341B cell line caused severe cachexia in tumor bearing mice. Similar symptoms have also been observed in mice injected with tumorigenic variants of WM35, another early stage melanoma cell line (unpublished observations). No such symptoms were observed in the case of naturally derived advanced melanoma xenografts including MeWo and several other cell lines (data not shown). The cachexia inducing factors are unknown at the current time, but tumor cell derived products (possibly TNFα) are more likely candidates than high systemic levels of VEGF/VPF.
VEGF appears to act as an indirect survival factor for the WM1341B cell line in vivo.

Immunohistochemical analysis revealed that the introduction of VEGF into the otherwise dormant WM1341B cell line shifted the balance of proliferation and death towards a net increase in growth, not by increasing the number of cells proliferating but rather by decreasing the extent of tumor cell death. In this study a limited number of slides were used for immunohistochemical analysis. More extensive immunohistochemical analysis including quantification of the data would be useful to build a more solid argument for this observation.

It was difficult to determine the type of cell death that the WM134B melanoma cells undergo in vivo. Staining with apotag, which selectively stains apoptotic cells, produced results which were hard to assess due to non-specific staining. The tumor sections appeared to have both apoptotic and necrotic populations. Nevertheless, regardless of the specific type of cell death that was present, it appeared that cells transfected with the VEGF gene differed in that they experienced less cumulative cell death (that being apoptotic or necrotic cell death) than the parental cell line but relatively the same extent of tumor cell proliferation. One may speculate that angiogenesis allows the cells to survive in vivo by relieving the tumor bed from hypoxia which can result in cell death and thus perhaps acts in an indirect manner as a survival factor for human melanoma. For the tumor, this would mean that once the angiogenic process was engaged (by the upregulation of VEGF or another stimulator of angiogenesis), there would be a net increase in tumor size. This pathway may behave as a positive feedback loop such that once angiogenesis is turned on in a dormant tumor, each increase in tumor size would result in the production of more VEGF which would stimulate angiogenesis and in turn allow the tumor mass to grow.
There is a lack of understanding of the mechanism through which melanoma cells undergo cell death. While in other cell systems, apoptotic cell death is has been shown to occur, the argument for apoptosis in melanoma is not yet convincing. Many investigators have been unable to detect the traditional features of programmed cell death in melanoma cell systems. That is not to say that there is no spontaneous programmed cell death, and indeed it appears that there are some evidence of apoptotic-like death present in melanoma, but the classical, definitive features of apoptosis such as DNA "laddering" or fragmentation, are not consistently present. In the WM1341B cell system, the abundant and unspecific staining of necrotic regions by Apotag did not permit me to define cell death as apoptosis or necrosis. Perhaps the type of cell death observed in melanoma represents a new, currently undefined form of cell death which may or may not be specific to this particular disease. Since the relevance of apoptosis to melanoma has been questionable, it may be worth redefining programmed cell death as it applies to this disease. Regardless of the lack of definition as to the specific type of cell death observed, there were clear differences in the extent of cell death. It is more likely that it is angiogenesis and not VEGF per se which is behaving as a survival factors for the melanoma cells. Thus, VEGF probably acts indirectly through stimulation of angiogenesis to allow survival of the cells, and does so presumably by the ability of the vasculature to perfuse the tumor mass and allowing the cells to be relieved from tumor associated hypoxia.

Even though overexpression of VEGF/VPF was clearly sufficient to induce an aggressive tumorigenic phenotype in WM1341B cells, the same is not necessarily true for other non-tumorigenic immortalized VEGF/VPF cell lines. As discussed above, expression of exogenous VEGF/VPF in VEGF/VPF negative and immortal Chinese hamster ovary cells led to the
formation of small benign outgrowths, rather than overt aggressive tumors (81). In the case of several clones of the immortalized (and VEGF/VPF negative) rat intestinal epithelial cell line called IEC-18 (149) induced to express high level of human VEGF/VPF$_{121}$, injection into nude mice failed to produce any signs of tumor growth (Rak et al. unpublished observation) even though mutant ras oncogene transfection of IEC-18 cells induces both VEGF/VPF and tumorigenic competence (90). This comparison reinforces the point that induction of an angiogenic phenotype is necessary, but not sufficient for expression of tumorigenic phenotype (35). For example, in many instances, transformed cells are simply unable to survive under three-dimensional conditions of the tumor (31). This is not the case for transformed WM1341B cells which appear to survive readily in three-dimensional culture, and which can remain viable for an extended period of time even after subdermal inoculation. Upon overexpression of VEGF/VPF in these cells, the pre-existing transformed status and angiogenic proficiency combine to result in an overt tumorigenic phenotype. In this case, angiogenesis prevents necrotic changes in the tumor mass, thus acting as a higher order, and indirect, survival factor.

The oncogenic changes which accompany melanoma progression may lead to greater inducibility of VEGF rather than constitutive overexpression of the protein.

In the cell lines examined, the relative levels of constitutive expression of VEGF did not necessarily correlate with the particular stage of melanoma progression from which the cell lines were derived. Instead, it was observed that upregulation of VEGF by hypoxia-like conditions appeared to correlate with melanoma progression. That is, cell lines from both late stage metastatic melanoma and matrigel induced tumorigenic variants of early melanoma seemed to
upregulate VEGF to a much greater extent under hypoxic conditions than did cell lines derived from early stage melanoma. This observation is based upon the few cell lines which were examined in this study. To put forth more conclusive evidence, this type of study should be repeated on a greater number of cell lines derived from the various stages of melanoma. Our results are consistent with those of others who have reported that the ability of hypoxia to upregulate VEGF expression is significantly enhanced in cells, such as NIH-3T3 fibroblasts, if the cells have a mutant oncogene and are transformed, i.e. there is a synergistic effect (150).

Although it was not examined in this study, it is likely that the same pattern of VEGF upregulation would hold true for other microenvironmental inducers of VEGF such as glucose deprivation and molecular upregulators of VEGF such as IL-6. This would mean that as a tumor advances from early stage to advanced stage melanoma, its ability to induce an angiogenic response upon stimulation would increase. For example, as melanoma is relieved from dormancy by the onset of angiogenesis, there will be an increase in size, and the demand for angiogenesis to alleviate symptoms of hypoxia would be satisfied by the tumor cells' ability to upregulate VEGF to a greater extent. A similar scenario could be applied to IL-6. IL-6 which is growth inhibitory for early stage melanoma, is secreted by endothelial cells. As tumor angiogenesis is engaged, cells which are resistant to IL-6 and can upregulate the production of VEGF by IL-6, will become the dominant population. Furthermore, as the tumor evolves, it may have increased VEGF upregulation and therefore angiogenesis in response to IL-6.

If the trend of greater inducibility of VEGF holds true when additional cell lines are screened for this feature, there could be some interesting applications of this observation, which may allow a greater understanding of the factors governing melanoma progression. In this
regard, abrogation of either the von Hippel Lindau (VHL) or the p53 tumor suppressor genes has been shown to enhance the tumor cells' ability to upregulate VEGF, especially under inducing conditions such as hypoxia (94,95). Thus, it is conceivable that the increase in the cells' ability to upregulate VEGF along the spectrum of melanoma progression may reflect changes in genes such as inactivation of p53 or VHL which may accompany and/or drive tumor progression. In this regard, little is known about the genetic changes driving melanoma progression and parallel changes in malignant angiogenesis. For this reason, it is particularly intriguing that in a clonally related pair of cell lines, i.e., non-tumorigenic (WM1341B) and tumorigenic (WM1341B-P3N2) cells, constitutive VEGF/VPF production was undetectable, whereas a significant difference was observed in VEGF/VPF inducibility by hypoxia. It is tempting to speculate that putative oncogenic changes that apparently occurred during the in vivo "Matrigel" selection of WM1341B cells for high grade tumorigenicity (119), might have affected in a selective way hypoxia-dependent mechanisms of VEGF/VPF gene transcription and/or mRNA stability. Such cell lines could potentially be used to uncover genes which are likely to accompany melanoma tumor progression.

The VEGF transfected WM1341B cell line may be a useful system by which to screen potential anti-angiogenic therapeutic agents.

The cell system which was developed in this study, comprised of the early stage WM1341B cell line and sublines which was transfected with VEGF121, may be used to test the activity of VEGF directed anti-angiogenic agents because these cell lines are dependent strictly upon VEGF-induced angiogenesis for their tumorigenic phenotype. Treatment with anti-
angiogenic agents, especially those directed towards inhibiting VEGF or VEGF receptors, should cause the cells to return to their dormant-like phenotype *in vivo*. The efficacy of the system was demonstrated by treating the transfected cell line with A.4.6.1 anti-VEGF neutralizing antibody. In this experiment, a significant reduction in tumor size was observed although complete dormancy phenotype of the parental cell line did not prevail. This most likely reflects the incomplete penetration of the antibody into the tumor mass, and/or the presence of mouse VEGF (the source of which may be tumor infiltrating stromal cells) which would not be neutralized by a mouse anti-human VEGF monoclonal antibody. Since antibodies are quite large molecules, it may be useful to develop other anti-VEGF compounds which may have better tumor penetration abilities. It would be interesting to compare the results of the A.4.6.1 antibody to the same system treated with other anti-angiogenic agents such as angiostatin (108) or endostatin (110).

Considering the conclusions discussed so far, anti-angiogenic therapy is likely to prove useful in melanoma tumor mass reduction and if used early enough in disease progression, may keep the tumor in a static i.e. dormant state. This makes sense based upon the principle that angiogenesis shifts the balance of proliferation and cell death towards proliferation and therefore a net increase in tumor growth. Inhibition of the angiogenic process probably maintains the tumor in a small, dormant state, where there is little hypoxia, glucose deprivation, and endothelial cell derived inhibitory factors such as IL-6. The proximity of the tumor cells to neighboring capillaries is most likely sufficient to meet the needs of the small, dormant tumor.

Experiments in which the A.4.6.1 antibody was used to treat mice injected with cell lines derived from metastatic melanoma or matrigel derived tumorigenic variants of early stage melanoma revealed that tumor reduction could be observed when the effects of VEGF were
neutralized. In such cell lines it would be interesting to compare the degree of tumor reduction when similar tumors are treated with either chemotherapy, anti-VEGF or an anti-angiogenic treatment such as angiostatin. Considering the ratio of tumor associated endothelial cell to tumor cell is in the order of 1:10 or 1:100 (114), treatment with anti-angiogenic therapy, which targets the endothelium, may be as efficient as, or more potentially more efficient than treatment with chemotherapy, which targets the tumor cells. Moreover, if anti-VEGF treatment or anti-angiogenic treatment can give similar or greater reductions in tumor size, it may be strongly support the use of this kind of treatment since it carries with it little side effects (41), and has thus far failed to induce drug resistance as is the case with conventional chemotherapy (41), both of which are commonly observed deleterious features of chemotherapeutic treatment. However, not all anti-angiogenic agents will bypass drug resistance. In theory resistance will not develop against drugs such as angiotatin or endostatin - which directly target genetically stable vascular endothelial cells. In contrast, anti-VEGF directed drugs actually target a property of the genetically unstable tumor cell population. One would expect that treatment with anti-VEGF antibodies would eventually result in the emergence of a VEGF-independent subpopulation of tumor cells which express other angiogenesis-inducing growth factors.

It would also be interesting to examine the effects of treating the tumors with both chemotherapy and anti-angiogenic therapy in combination. Considering the notion that the two populations of cells - the endothelium and the tumor cells - have a symbiotic-like relationship, it is likely that combination treatment would result in the synergism of both therapies (49,114).
Summary

In the context of what is currently known about melanoma progression, the results from this study suggest that VEGF induced angiogenesis can rescue early stage melanoma cells from tumor dormancy, at least in the case of the WM134B cell line. Although this study has its limitations, it certainly builds a strong argument for the onset of the angiogenic process as being a key player in the progression of melanoma from early to late stage primary disease. It will be important in the future to carefully examine the various aspects of the regulation of the angiogenic process in melanoma and other malignant diseases, as solid tumors are known to be strongly dependent upon the angiogenic process for tumor growth (4,30). This study suggests that targeting angiogenic factors, including VEGF, may have important applications in cancer therapy, particularly towards melanoma, for which there is currently no effective treatment when in its more advanced stages of disease progression.
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