Vascular Endothelial Growth Factor Isoform Expression as a Determinant of Blood Vessel Patterning in Human Melanoma Xenografts

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Abstract

Vascular endothelial growth factor (VEGF) occurs in at least five different isoforms because of alternative splicing of the gene. To investigate the roles of different VEGF isoforms in tumor blood vessel formation and tumorigenicity, the three major isoforms (VEGF121, VEGF165, and VEGF189) were overexpressed in an early-stage human melanoma cell line (WM1341B), which is VEGF-negative and nontumorigenic in immunodeficient mice. Although overexpression of VEGF121 and VEGF165 resulted in aggressive tumor growth, WM1341B cells transfected with VEGF189 remained nontumorigenic and dormant on injection. Although tumor growth rate depended on the level and not the isoform of VEGF expressed, striking isoform-specific differences in vascular patterning were associated with VEGF121 versus VEGF189-dependent tumorigenic conversion of human melanoma. Thus, tumors overexpressing VEGF165 generated dense, highly heterogeneous vessel networks that were distinctly different from those of tumors expressing VEGF121 (poorly vascularized and necrotic). Paradoxically, although VEGF146 expression appears to result in the most effective tumor perfusion, it is the expression of VEGF121 that is observed during human malignant melanoma progression. Indeed, unbiased selection of spontaneously tumorigenic variants of WM1341B (by coinjection with Matrigel) led to predominant expression of the VEGF121 isoform. The vascular patterning in these tumors (1341-P3N1, 1341-P3N2) resembled that of the VEGF121-transfected WM1341B tumors. These results suggest that, for reasons that remain to be elucidated, a “minimal” program of tumor vascularization may be favored during melanoma progression.

Introduction

VEGF, also known as vascular permeability factor (1, 2), is an endothelial cell-specific growth factor and the principal regulator of angiogenesis under both normal and pathological conditions such as tumor growth. VEGF is overexpressed in the vast majority of human tumors (3), including malignant melanoma (4). The expression of VEGF is subject to complex regulation as alternative splicing of the human VEGF gene results in at least five isoforms containing 121, 145, 165, 189, or 206 amino acids (the corresponding murine isoforms are one residue shorter). Because of differential incorporation of the basic residues encoded by exons 6 and 7, VEGF isoforms differ in their heparin-binding properties, membrane association, and secretion. VEGF121, which lacks the basic residues of both exons, does not bind heparin-containing cell surface proteoglycans, and is freely soluble. VEGF165 is also secreted; however, cationic residues in exon 7 enable it to bind heparin, and, thus, some remains bound to the cell surface or extracellular matrix (5). The largest isoform, VEGF189, which retains both exons, has the highest affinity for heparin and, therefore, remains tightly cell associated. However, shorter bioactive forms of VEGF189 (e.g., VEGF110) are released on cleavage by proteases such as plasmin (6) and urokinase plasminogen activator (7).

Perhaps the most compelling evidence for isoform-specific biological functions has been provided by recent gene-targeting studies. Mice that expressed the VEGF120 isoform, but lacked the 164- and 188-residue splice variants, exhibited impaired myocardial angiogenesis and ischemic cardiomyopathy, which led to death (8). The notion of distinct isoform functions is further supported by the existence of isoform-specific interactions with VEGFRs. All of the isoforms of VEGF bind to two related endothelial cell-specific receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flik-1; Refs. 9, 10); however, VEGFR-2 binding is enhanced by a non-kinase coreceptor, neuropilin-1 (NP-1), which specifically binds to exon 7-encoded sequences found in VEGF165 but not in VEGF121 (11). Additional isoform-specific VEGFRs may also exist (12, 13).

The widely held view that VEGF165 is the predominant isoform expressed (3) is not always justified. For example, VEGF189 is the major isoform expressed in the lung (14, 15), whereas both VEGF165 and VEGF189 predominate in the heart (8). Other tissue-specific differences in the spatial distribution of expressed VEGF isoforms have also been reported (15–17). Furthermore, the relative levels of the VEGF isoforms not only differ from organ to organ but may also vary during development (15).

VEGF isoform expression also varies in cancer. For example, VEGF121 appears to predominate in melanoma (4, 18) and in breast carcinoma (19), whereas VEGF165 is the major splice variant present in glioblastoma (20). Cheung et al. (14) showed that in non-small cell lung carcinoma, malignant progression was associated with a switch from a VEGF189-rich expression pattern to the smaller isoforms, and others have detected changes in VEGF splicing during skin carcinogenesis in the presence of the v-H-ras oncogene (21). VEGF splicing represents a level of VEGF regulation that occurs actively, both normally and in the context of cancer. However, this process remains poorly understood.

As a first step to investigate why progression of certain tumors may favor one VEGF isoform over the other, we stably transfected the WM1341B human melanoma cell line with the three major isoforms: VEGF121, VEGF165, and VEGF189. Onset of angiogenesis and VEGF expression are associated with the transition from avascular early-stage melanoma to the poorer prognosis vertical-growth phase (22, 23) and, indeed, may play a causal role in tumor progression. For example, the WM1341B cell line, which was derived from a very early-stage primary melanoma, fails to grow when inoculated into immune deficient mice, even after orthotopic injection of large cell numbers (24). However, coinoculation of the cells with Matrigel, a proangiogenic basement membrane extract (25), resulted in rapid tumor formation (26). This suggests that the defect in WM1341B tumorigenicity may be attributable to a deficiency in expression of certain proangiogenic growth factors that are turned on at later stages of melanoma progression. Indeed, WM1341B cells do not express VEGF mRNA or protein in culture, even under hypoxic conditions (24).
We reasoned that if WM1341B cells represent a preangiogenic stage of development of malignant melanoma, it would constitute an ideal model for testing the functional consequences of experimental induction of the angiogenic phenotype, in particular, expression of various VEGF isoforms. It could be predicted that because VEGF_{121} is most abundant in melanoma, expression of this isoform should lead to the most effective tumor vascularization. Contrary to this prediction, our study showed that enforced expression of VEGF_{185} in WM1341B cells is associated with greater tumor vascularity than in the case of VEGF_{121} transfectants. WM1341B cells expressing VEGF_{189} remained angiogenesis incompetent and nontumorigenic. Furthermore, marked qualitative differences were noted in three-dimensional vascular patterning and architecture associated with VEGF_{121} versus VEGF_{185}-dependent tumorigenic conversion of human melanoma.

MATERIALS AND METHODS

Cell Culture and Cell Lines. The WM1341B, WM35, WM115, WM239, WM451, and WM9 human melanoma cell lines were kindly provided by Dr. Meenhard Herlyn (Ref. 27; Wistar Institute, Philadelphia, PA). All of the melanoma cells were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 5% fetal bovine serum (Life Technologies, Inc.).

Transfection and Selection. The human VEGF_{185} expression construct was a gift from Dr. Shay Soker (Harvard Medical School, Boston, MA). The expression construct for VEGF_{189} (28) was kindly provided by Dr. Webster Cavenee (Ludwig Institute for Cancer Research, University of California at San Diego, La Jolla, CA). For each construct, the inserts were removed and the vector was used in control transfections. Briefly, 200,000 cells were plated overnight, then transfected with 2 µg of DNA using Lipofectin (Life Technologies, Inc.), as instructed by the supplier. For the VEGF_{185} transfections, neomycin-resistant colonies were selected in complete growth medium containing 400 µg/ml Gentamicin (Life Technologies, Inc.). The VEGF_{189} expression construct contained a hygromycin-resistance cassette, and transfected cells were selected in standard culture medium containing 100 µg/ml hygromycin (Boehringer Mannheim). Two individual clones of VEGF_{121}-transfected WM1341B were generated as described previously (24).

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from transfected cells using Trizol (Life Technologies, Inc.) as described by the manufacturer. Forty µg of total RNA was subjected to electrophoresis on a 1% agarose gel, and ethidium bromide staining was performed to visualize 18S and 28S rRNA and control for RNA loading. The RNA was transferred to Hybond-N+ nylon transfer membrane (Amersham Pharmacia Biotech, Baie d’Urfe, Quebec, Canada), and the blots were hybridized with a 200-bp VEGF probe.

Measurement of Human VEGF Protein Levels in Conditioned Medium by ELISA. A commercially available ELISA kit (R&D, Minneapolis, MN) was used to quantify VEGF protein levels in conditioned medium. Cells were plated in triplicate, overnight in 24-well plates, at a density of 2.5 × 10^6 cells/well, followed by the addition of fresh serum-free medium. To release cells, 0.5 ml of 0.05% trypsin (GIBCO-BRL) was added, and the cells were collected by centrifugation. The cell pellets were washed twice with PBS, followed by a wash with PBS containing 0.5% FBS. The cells were lysed by addition of 0.5 ml of 1% Triton X-100, followed by 0.5 ml of 5X LDS sample buffer (100 mM Tris-HCl (pH 6.8), 0.05% SDS, 0.0005% bromophenol blue, 5% β-mercaptoethanol). The lysates were heated at 95°C for 10 min, and separated by SDS-PAGE, before being transferred to a nitrocellulose membrane. The membrane was probed with a rabbit polyclonal antibody to human VEGF (1:500 dilution) and then with a horseradish peroxidase-conjugated secondary antibody. The bands were visualized using chemiluminescence detection reagents (Amersham Pharmacia Biotech). The intensity of the bands was quantified using the NIH Image software.

RT-PCR Analysis of VEGF Isoform Expression Pattern. Ten µg of RNA was reverse-transcribed into cDNA in a 20-µl reaction mixture with 100 pmoles of oligo-dT12 primer, 0.5 mM dNTP, 100 units Moloney Murine Leukemia Virus (MMLV)-reverse transcriptase (Stratagene, La Jolla, CA), and the reaction buffer provided by the supplier, for 1 h at 37°C. Three µl of the reaction product was used in subsequent PCR reactions containing 800 nm primers, 0.2 mM dNTP, and 5 units Taq polymerase (Amersham Pharmacia Biotech) in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA). VEGF isoforms were amplified by 30 cycles of PCR consisting of 1 min at 94°C, 2 min at 64°C, 3 min at 72°C, followed by 3 min at 72°C, using previously described primers (29) as follows: 5′-CTG CTT GCT GCT CTA CCT CC-3′ (forward) and 5′-TCA CGG CCT CGG CTT GTG AC-3′ (reverse). After electrophoresis on 2% agarose gels, the different VEGF isoforms could be discriminated based on the sizes of the RT-PCR products: amplification of VEGF_{121}, VEGF_{165}, and VEGF_{189} produced bands of 410, 540, and 610 bp, respectively. The 480-bp and 660-bp products corresponding to VEGF_{165} and VEGF_{189} were not observed in any of the melanoma tumor cells examined. As a control, the housekeeping GAPDH gene was amplified using the following primers: 5′-GCA GGG AGG AGC CAA AAG GG-3′ (forward) and 5′-TGC CAG CCC CAG CGT CAA AG-3′ (reverse).

Tumorigenicity Assay. To assess tumorigenicity, 5 × 10^6 melanoma cells were injected orthotopically (30) into the subdermal region in the flanks of 4- to 6-week-old CB-17 SCID mice (Charles River, St. Constant, Quebec, Canada). Tumor volume (mm^3) was estimated using the standard formula (length × width^2 × 0.5). When tumors reached a volume of ~100 mm^3, vascular patterning was analyzed by FITC-lectin injection, vascular casting or immunostaining, as described below. Smaller and larger tumors were also examined in each case for comparison. For analysis of patterning, a minimum of six different tumors was examined for each of the VEGF_{121} and VEGF_{185} transfected WM1341B clones.

Intravascular FITC-Lectin Injection for Three-Dimensional Detection of Tumor Vessels. Mice were anesthetized with 50 mg/kg sodium pentobarbital (MRB Pharmaceuticals, Cambridge, Ontario, Canada) injected i.p. and FITC-labeled Lycopersicon esculentum lectin (Vector Laboratories, Burlingame, CA; Ref. 31) injected into the tail vein (100 µg in 200 µl of 0.9% NaCl). After 5 min, the chest was opened quickly, and tissues were fixed by perfusion of 0.5% glutaraldehyde and 1% paraformaldehyde in PBS from an i.v. catheter (Becton Dickinson, Sandy, UT) inserted into the left ventricle (31). The vasculature was flushed at a pressure of 100 to 120 mm Hg until washed free of blood. Tumors were dissected, rinsed in PBS, and cryoprotected for 1 h at 4°C with 15% sucrose in PBS, followed by 30% sucrose in PBS overnight at 4°C. Tissue was placed in Tissue-Tek OCT Compound (Miles Inc., Elkhart, IN) at 4°C for 1 h before freezing over dry ice. Cryosections of 100 µm were cut, placed on silanized slides, coverslipped with Aqua Poly/Mount (Polysciences, Warrington, PA), and viewed under fluorescence confocal microscopy to visualize intravascular FITC-labeled lectin.

Microvascular Corrosion Casting. Mice were anesthetized by i.p. injection of 50 mg/kg sodium pentobarbital (MRC Pharmaceuticals), injected i.v. with 10,000 units/kg heparin (Wyeth-Ayerst, St. Laurent, Quebec, Canada) via the tail vein. The chest was opened, and the vasculature was rinsed with lactated Ringer’s solution (Baxter, Toronto, Ontario, Canada) plus 1 unit/ml heparin until clear of RBCs, by means of an i.v. catheter (Becton Dickinson) inserted into the left ventricle of the heart. After complete flushing, mice were perfused with Batson’s no. 17 casting polymer, a mixture of 6.5 ml monomer base, 1.5 ml catalyst, and 0.2 ml promoter solution (Polysciences, Warrington, PA; Ref. 32). Pressure perfusions were controlled manually and ranged from 100 to 120 mm Hg during the procedure. After polymerization, the tissue was digested by placing the entire mouse in a 30% potassium hydroxide solution, followed by washing with warm running tap water; this was repeated until maceration was complete. The tumor vessel casts were removed, rinsed in distilled water, and dried. For scanning electron microscopy, tumor vessel casts were mounted on specimen holders and sputter-coated with gold in an argon atmosphere. Vessel casts were examined with a Hitachi S-570 scanning electron microscope at an acceleration voltage of 15 kV and working distance of 15 mm.

Immunohistochemistry. Tumor tissue samples were rinsed in PBS and cryoprotected in 15% sucrose in PBS followed by 30% sucrose in PBS at 4°C. Tissue was placed in Tissue-Tek OCT Compound (Miles Inc., Elkhart, IN) at 4°C for at least 1 h before freezing over dry-ice. For CD31 immunostaining, cryosections of 5 or 10 µm were cut and incubated with a rat antimonkey CD31 antibody (PharMingen, San Diego, CA) diluted 1:200, followed by a biotinylated rabbit antirat IgG (Jackson Laboratories, West Grove, PA) and streptavidin peroxidase conjugate (Zymed, San Francisco, CA). The peroxidase reaction was developed with 3-amino-9-ethylcarbozole (Zymed), and slides were counterstained with hematoxylin.

RESULTS

VEGF Expression in Transfected WM1341B Cells. The WM1341B cell line was derived from a patient with an early-stage primary human melanoma lesion who was cured as a result of the

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surgery (27). These cells were chosen for VEGF transfection because they are nontumorigenic and constitutively express negligible amounts of VEGF mRNA and protein, which are changed only marginally by hypoxia (24). WM1341B cells also do not produce basic fibroblast growth factor as measured by ELISA of cell lysates, nor express angiopoietin-1, as determined by RT-PCR (data not shown). Therefore WM1341B cells were stably transfected with constructs encoding VEGF121, VEGF165, or VEGF189 cDNA under the control of a CMV promoter. Two of the 11 VEGF121-transfected clones (121-2 and 121-24; Ref. 24), were used in this study. Nine VEGF165-overexpressing WM1341B clones were obtained, and the two highest expressors, 165-24 and 165-20, were chosen for further analysis of vascular patterning. Three clones transfected with VEGF189 were also obtained. Successfully transfected clones were readily identified by the presence of a unique exogenous 1.9-kb mRNA band on Northern blots (Fig. 1A). This band was not observed in cells transfected with an empty vector. As expected, the 4.2- and 3.7-kb bands corresponding to endogenous human VEGF mRNA were nearly undetectable in the parental and transfected WM1341B cells.

Secretion of VEGF protein into the conditioned medium was assayed using a VEGF-specific ELISA. As shown in Fig. 1B, the 165-24 and 165-20 VEGF165-transfected clones secreted high levels of VEGF, whereas the parental WM1341B cell line did not express detectable levels of VEGF protein. The level of VEGF protein secreted by the VEGF121-transfected 121-2 clone was comparable with one of the VEGF165-transfected clones (165-20), and, thus, comparisons with respect to vascular patterning could be made with these clones in particular. As expected, the VEGF189 protein produced by VEGF189-transfected clones was not detectable in the conditioned medium, because this isoform is not secreted but remains cell-associated (5, 6). However, the addition of heparin to the medium of the VEGF189-transfected cells released this fraction and increased the detected VEGF concentration 9.19 ± 0.28-fold for clone 189-1. In comparison, heparin treatment of VEGF121-expressing cells did not significantly increase the VEGF level detected in the conditioned medium (1.23 ± 0.02-fold increase for clone 121-2). A small increase was seen in the case of the 165-20 VEGF165-transfected clone (2.30 ± 0.07-fold), reflecting the partial retention of this isoform in the extracellular matrix. Overexpression of VEGF had no effect on basic fibroblast growth factor levels produced by transfected clones (measured by ELISA of cell lysates, data not shown), and, therefore, the observed properties of these cells can likely be attributed to the effect of VEGF isoform expression.

**Tumorigenicity of VEGF-transfected WM1341B Cells.** To examine the effects of VEGF transfection on tumor growth in vivo, VEGF165-and VEGF189-overexpressing WM1341B cells were injected orthotopically (subdermally) into SCID mice (30). As shown in Fig. 1C, clones expressing VEGF165 were highly tumorigenic, and the VEGF121-transfected WM1341B cells also produced rapidly growing tumors in mice (24). For VEGF121 and VEGF165 overexpressors, the rate of tumor growth correlated with the amount of VEGF protein produced by each clone rather than by the isoform expressed. In contrast, it was found that all three clones overexpressing VEGF189 (189-1, 189-8, 189-16) were nontumorigenic and, similar to the parental WM1341B cells, did not give rise to visible tumors even after more than 210 days of observation. Any conceivable autocrine effects of VEGF (33) were ruled out on the basis of the following observations. First, overexpression of the VEGF isoforms did not have a significant effect on the in vitro growth rates of WM1341B cells in monolayer culture (data not shown). Second, these cells did not express VEGFR-2 (24) or neuropilin-1 (data not shown). Finally, proliferation of VEGF121 transfectants in vitro is not affected by treatment with a neutralizing anti-VEGF antibody (24). Therefore, it was concluded that, in all likelihood, the tumorigenicity of the VEGF121 and VEGF165 transfectants could be attributed to a stimulation of angiogenesis.

**Vascular Patterning in VEGF121- versus VEGF165-Overexpressing Tumors.** Although the overall growth rate of the tumor mass was correlated with levels of secretable VEGF rather than with a specific VEGF isoform, dramatic and qualitative differences in VEGF165- versus VEGF121-induced three-dimensional vascular patterning were observed using several different approaches. First, a method of intravascular FITC-lectin injection was used to visualize the tumor vessels (31). This involved i.v. injection of fluorescent L. esculentum lectin, which subsequently becomes bound to the luminal surface of blood vessels. After perfusion fixation of the tissues, examination of thick 100-μm tumor cryosections using fluorescence confocal microscopy provided excellent three-dimensional images of the clearly labeled tumor vasculature. To confirm the vessel pattern shown by fluorescent lectin staining, endothelial cells in both 100-μm and 7-μm tumor cryosections were localized by CD31 immunohistochemistry. Finally, truly three-dimensional visualization of tumor

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**Fig. 1.** Expression of VEGF165 but not VEGF189 rescues the dormant phenotype of early-stage WM1341B melanoma cells. A. Northern blot analysis of exogenous (1.9 kb) and endogenous (4.2 and 3.7 kb) human VEGF mRNA expression. B. Secretion of VEGF protein into conditioned medium by transfected clones, as measured by ELISA. Concentrations given in pg/ml are per 2.5 × 10^7 cells/24 h. C, tumor volume 22 days after orthotopic injection of 5 × 10^6 melanoma cells in SCID mice. Values to the right, tumor take (number of tumors/number of mice injected). Overexpression of each VEGF isoform had no significant effect on cell growth rates in vitro (data not shown).
Vessel architecture was achieved by microvascular corrosion casting of tumors. VEGF121 and VEGF165-transfected clones producing similar amounts of VEGF (121-2 and 165-20) were selected for this analysis (Figs. 2–4). Compared with VEGF121-transfectant tumors, VEGF165-overexpressing tumors were significantly more vascularized. As shown in Fig. 2, A and C, VEGF165 tumors contained regions of intense fluorescent lectin-bound and CD31-positive disorganized vasculature surrounding nodules of tumor cells. In remarkable contrast, such extensive vessel networks were not observed in VEGF121-driven tumors (Fig. 2, B and D). When thin 7-μm CD31-immunostained sections were examined, it became clear that the variably sized nodules of VEGF165-overexpressing tumor cells were surrounded by abundant thin-walled endothelialized blood spaces that, for the most part, were abnormally large in size and that ranged greatly in diameter (Fig. 2, E and G). In contrast, the sparsely distributed vessels resulting from expression of VEGF121 in tumor cells appeared much less irregular in diameter, with sprouts and loops often observed. Scale bars: A and B, 500 μm; C and D, 100 μm; E and F, 175 μm; G and H, 50 μm.

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and the “holes” as well as the vessels themselves varied in diameter (Fig. 4A). Closer examination of the CD31-immunostained sections showed that CD31-positive endothelial cells often extended into the lumina of larger vessels and formed bridges dividing the lumen into multiple smaller channels (Fig. 4B). Such thin bridges could also be visualized in the lectin-stained tumor sections (Fig. 4C). These features appeared to be specific to tumors derived from the VEGF165-overexpressing WM1341B cells. Combining the information from CD31 and lectin staining, the small holes seen in the vascular casts might, therefore, be indicative of the invasion of endothelial cell processes into the lumen. Further recruitment of endothelial cells might result in widening and stabilization of these divisions (seen as enlargement of holes on vessel casts), and consequently in the expansion of the vessel network by bridging and intussusception (34), resulting in the dense vascularization observed in VEGF165 but not VEGF121 tumors.

**Predominant Expression of VEGF121 by Both Early-Stage and Advanced Melanoma Cell Lines.** Next we examined the VEGF isoform expression profile of a panel of melanoma cell lines using a RT-PCR assay that yielded products of 410, 540, and 610 bp corresponding to amplification of VEGF121, VEGF165, and VEGF189, respectively. As a confirmation of isoform identity, the expected splice variants were detected in the WM1341B clones transfected with each of the respective VEGF isoforms (Fig. 5A, Lanes 1–3). Analysis of cell lines derived from early-stage, as well as from advanced or metastatic melanoma lesions, showed that whenever VEGF expression was detected, and independent of the stage of tumor progression, VEGF121 was the predominant isoform expressed. Although the WM1341B cell line used in the transfection experiments produced negligible amounts of VEGF mRNA (Fig. 1, A and B), amplification with the highly sensitive RT-PCR assay revealed that, similar to other melanoma cell lines, the small amount of endogenous VEGF that was detectable was predominantly VEGF121, with a much lower level of VEGF165.

**Vascular Patterning and VEGF Isoform Expression of a Tumorigenic WM1341B Variant.** When we considered the greater vascularity that was observed in the VEGF165-overexpressing WM1341B tumors, we expected that this isoform should be favored on VEGF up-regulation during the progression of WM1341B melanoma. To determine whether this was in fact the case, we examined two individual tumorigenic variants of WM1341B derived by serial in vivo passage with Matrigel (26). These tumorigenic sublines of WM1341B (named WM1341-P3N1 and P3N2) no longer require Matrigel support for in vivo growth and angiogenesis, and recapitulate many properties of a more advanced melanoma in terms of cellular responses to growth factors and cytokines. For example, they are relatively resistant to interleukin-6-induced growth inhibition (26). These cell lines also produce significant levels of VEGF that are
readily inducible by hypoxia (Fig. 5B), and the relative tumor growth of these variants is illustrated in Fig. 1C. Somewhat unexpectedly, overexpression of VEGF121 is also favored during spontaneous tumorigenesis of the WM1341B cell line. This isoform was predominant in both P3N1 and P3N2 cell lines as well as in tumors in vitro (Fig. 5C). Furthermore, when WM1341-P3N1 and P3N2 tumors were examined by intravascular lectin binding and CD31 immunohistochemistry, the vasculature resembled that of the parental WM1341B cell line engineered to overexpress VEGF121, and was markedly different from that of the hypervascular VEGF165-overexpressing WM1341B tumors (Fig. 5, D–F). In both P3N1 and P3N2 tumors, vascularization was sparse, and necrotic areas apparent throughout the tumor.

DISCUSSION

Although the importance of VEGF as a tumor angiogenesis factor has been well established, the functional significance and the regulation of its splice isoforms in specific types of cancers remains poorly understood. In this regard, our study, which focused on malignant melanoma, contains several important findings. First, we have found that, whereas VEGF121 and VEGF165 overexpression convey tumorigenic competence to preangiogenic, early-stage WM1341B human melanoma cells, VEGF189 transfection does not. Second, whereas the growth of melanoma proceeds in a manner dependent on the level and not the isoform of VEGF expressed, the type of angiogenesis induced by VEGF165 and VEGF121 is vastly different. VEGF165 induced disorganized and dense vascular networks in well-perfused tumors. In contrast, VEGF121 induced a sparser, peripheral tumor vascularization, and the increase in tumor mass under the influence of various levels of VEGF121 occurred with a significant accumulation of necrotic areas. Third, although vascular patterning under the influence of VEGF165 appeared to result in more efficient tumor perfusion (minimal presence of necrotic areas), paradoxically it is the overexpression of VEGF121 that is selected for during spontaneous melanoma progression to an angiogenic and tumorigenic phenotype. Not only do naturally derived, advanced human melanoma cell lines preferentially express this VEGF isoform, but, moreover, the unbiased selection of angiogenic and tumorigenic variants of WM1341B also leads to selective expression of VEGF121. Thus, for reasons that remain to be elucidated, melanoma progression may favor a “minimal” program of tumor vascularization.

Differential Effects of VEGF Isoforms on Tumor Vessel Patterning. The microvascular pattern in a tumor is governed, at least in part, by the profile of angiogenic factors produced by the tumor cells, and may be characteristic of the tumor type (35). However, it is not clear how such seemingly simple biochemical signals elicited by such growth factors in target endothelial cells translate into the formation of complex, multicellular three-dimensional patterns. Despite the di-
agnostic and prognostic relevance of understanding what governs the spatial organization of vascular networks, few other studies have ever investigated the question of how tumor expression of a particular angiogenic factor may result in the generation of a certain microvascular pattern (36, 37). In particular, the role of different VEGF isoforms in tumor growth has been difficult to establish, perhaps because of the difficulty of evaluating the actions of a single isoform in isolation, given that the majority of tumors express multiple angiogenic growth factors (38). From this point of view, the WM1341B cell line used in this study represents an ideal and unique system, as angiogenesis in the tumors derived by VEGF transfection was driven essentially by either VEGF₁₂₁ or VEGF₁₆₅ alone. This made it possible to examine the direct effects of these isoforms on the generation and patterning of blood vessels in these tumors in a clinically relevant human tumor cell line.

Although the selected VEGF₁₂₁ and VEGF₁₆₅-overexpressing WM1341B cells secreted similar amounts of VEGF (121-2 and 165-20) and did not differ significantly in tumor growth rate, remarkable differences in vascular patterning were observed. These differences might arise firstly from the biodistribution of the isoforms, i.e., VEGF₁₂₁ is freely diffusible, whereas VEGF₁₆₅ is partially retained in the extracellular matrix. This may influence the rate of growth factor release and formation of concentration gradients, which might provide different guidance cues for mobilized endothelial cells. Secondly, the interactions of the isoforms with receptors that mediate VEGF signaling might be isoform-specific. Although both isoforms bind VEGFR-2 with equal affinity, the affinity of VEGF₁₆₅ for VEGFR-1 (Flt-1) is 20-fold higher than that of VEGF₁₂₁ (39). Furthermore, the VEGF₁₆₅-specific coreceptors neuropilin-1 and neuropilin-2 would be candidates for participation in such a mechanism. Unlike VEGF₁₂₁, the larger VEGF isoforms are able to interact with cell surface and extracellular matrix-associated heparan sulfate proteoglycans, e.g., glypican-1 binds VEGF₁₆₅ via its heparan sulfate chains (40). It is possible that these heparan sulfate proteoglycans might mediate other signaling events specifically downstream of VEGF₁₆₅-binding.

It should be noted that the very large caliber vessels observed in VEGF₁₆₅-transfected WM1341B tumors were reminiscent of the enlarged, thin-walled “mother vessels” described by Pettersson et al. (41) after adenoviral delivery of VEGF₁₆₅ to various normal adult mouse tissues. Such mother vessels were reported to undergo a variety of transitions, including sprouting and the formation of daughter vessels by intraluminal bridging initiated by the projection of activated endothelial cell processes into the vessel lumen. Interestingly, in our study, the examination of microvascular casts as well as the histology of VEGF₁₆₅-overexpressing tumors provided some evidence for both of these processes in WM1341B tumors.

It is not likely that the differential vascular architecture observed in the VEGF₁₂₁ versus VEGF₁₆₅-overexpressing tumors is attributable to clonal variation, because the characteristic patterns were observed reproducibly in tumors derived from two individual VEGF₁₂₁-transfected and two individual VEGF₁₆₅-transfected clones of WM1341B. Although the 165-24 clone produced 5-fold higher levels of VEGF than the 165-20 clone, tumors derived from both clones showed a similar vascular pattern despite differences in growth rate in vivo. Furthermore, the levels of VEGF secreted by the 121-2 clone were comparable with those secreted by the 165-20 lower expressor, as measured by ELISA. Thus, the differences in patterning were not related to differences in the level of VEGF expression, or the rate of tumor growth. Moreover, no significant differences were noted between smaller and larger tumors in each group, consistent with the previously described independence of blood vessel architectural properties from the size and rate of tumor growth (35, 36). Because VEGF₁₆₅ is partially cell associated, it would have been ideal to match the levels of total (secreted and cell-associated) VEGF in the clones. However, given our observation that the strongly cell-associated VEGF₁₆₅ was not able to confer tumorigenicity on WM1341B cells, the secreted forms of VEGF may play a more active role in vascularization than the extracellular matrix-sequestered fraction in this system. Despite this point, the matching of secreted VEGF may represent a limitation of our study.

Differential Capacity of VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ to Promote Tumor Growth. Unlike the smaller secretable isoforms of VEGF, VEGF₁₈₉ was unable to rescue the angiogenesis-deficient phenotype and promote the tumor growth of WM1341B cells. It is possible that this deficiency is a result of strong heparin-binding affinity and sequestration of this particular isoform at the cell surface, making it unavailable for activity (5). Our findings are consistent with a recent study in which single VEGF isoforms were expressed in VEGF-null but weakly tumorigenic marine embryonal fibroblasts transformed with the H-ras oncogene (42). It was found that expression of VEGF₁₆₅ was more effective in promoting tumor growth than was VEGF₁₂₁, and VEGF₁₈₉ was not effective at all. Such observations would suggest that the preponderance of the “more angiogenic” VEGF₁₆₅ should be expected in most tumors. Whereas this notion of more effective tumor vascularization under the influence of VEGF₁₆₅ holds true in overexpression studies with certain human tumors, including preangiogenic, nontumorigenic melanoma, natural selection favors or permits preferential expression of the VEGF₁₂₁ isoform in this type of malignancy.

VEGF Splicing in Human Melanoma. Our finding of predominant VEGF₁₂₁ isoform expression in human melanoma is consistent with previously published studies (4, 18), and could in theory result from two different influences on the splicing process: (a) the impact of malignant transformation during melanoma progression, or (b) factors involved in VEGF splicing (or splicing in general) that are specific to the melanocytic lineage. It is interesting to note that the relatively sparse vascularization and necrosis observed in the spontaneously derived WM1341B melanoma tumors is remarkably similar to the distribution of blood vessels found in clinical specimens of advanced melanoma (43, 44). To address the first hypothesis, selective expression of VEGF₁₂₁ (despite this isoform being less efficient in inducing angiogenesis than is VEGF₁₆₅) might suggest that extreme hypervascularity does not necessarily provide a stimulus for rapid tumor progression in this tumor type. Although microvessel counts have been used to establish angiogenesis as an independent prognostic indicator in various types of cancers, including breast (45, 46), prostate (47), and lung carcinomas (48), its prognostic significance for melanoma remains controversial because of conflicting published reports (43, 44, 49, 50). Furthermore, increased tumor hypoxia (exacerbated by low vascular density) has been shown to be associated with accelerated malignant changes and poor prognosis in a variety of cancers (51), and such a mechanism may contribute to the observed clinical malignancy and treatment resistance of human melanoma. Indeed, we have previously found that aggressiveness of melanoma growth in vivo often correlates with lowered “vascular dependence,” i.e., the ability to survive and expand at a lower blood vessel density (52). It is likely that the rapid tumor growth despite low vascular density, which was observed in the P3N1 and P3N2 variants of WM1341B melanoma, may reflect such a tendency.

Although there is a precedent for splicing changes associated with malignant progression, e.g., the switch from a VEGF₁₈₀-rich expression pattern in the normal lung to VEGF₁₂₁ and VEGF₁₆₅ in non-small cell lung carcinoma (14), additional studies are required to distinguish whether the expression of VEGF₁₂₁ is, in fact, actively selected during malignant transformation of melanocytes, or whether it is determined by lineage-specific factors. This issue will be difficult
to address because normal human melanocytes do not produce VEGF (18, 33).

In conclusion, our study suggests that melanoma progression is associated with the onset of angiogenesis using a tumor-specific profile of VEGF isoforms. The splicing of VEGF in melanoma cells may represent either a transformation-related or a lineage-specific event; and, in the context of the first possibility, tumorigenic conversion of melanoma can occur based on a minimally angiogenic phenotype and possibly through compensatory changes in the intrinsic resistance of tumor cells to ischemia (i.e., decrease in vascular dependence). A better understanding of VEGF splicing is necessary, because VEGF isoforms may prove to have prognostic relevance (53–55) and, possibly, implications for the design of new antiangiogenic therapeutics that target VEGF and its receptors (56, 57).

The results of our study serve to stress the importance of elucidating tumor progression-related and tumor-specific factors that regulate VEGF isoform splicing and, thus, the topology of the tumor vasculature.

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REFERENCES


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