Tumor-derived Expression of Vascular Endothelial Growth Factor Is a Critical Factor in Tumor Expansion and Vascular Function

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ABSTRACT

There is considerable controversy concerning the importance of tumor-derived angiogenic factors to the neovascularization of solid tumors. Tumor, endothelial, and stromal expression of vascular endothelial growth factor (VEGF) have been hypothesized to be critical for tumor angiogenesis. To determine the relative contribution of tumor versus nontransformed tissue expression of VEGF to tumor growth, we used gene targeting and cre-loxP recombination to generate embryonic stem cell lines in which VEGF can be conditionally deleted. These lines were used to derive mouse embryonic fibroblast lines with null mutations in both alleles of VEGF. Upon immortalization and H-ras transformation, we used these VEGF null fibroblasts to make fibrosarcomas in immunocompromised mice. We report that tumorigenic VEGF expression is critical for ras-mediated tumorigenesis, and the loss of tumorigenic expression causes dramatic decreases in vascular density and vascular permeability and increases in tumor cell apoptosis.

INTRODUCTION

The formation of new blood vessels is essential for tissue growth and expansion inasmuch as an ordered vasculature must be in place to provide oxygen and nutrients concurrently with significant increases in the cell number of solid tissues (1, 2). Angiogenesis is regulated by a number of both stimulating and inhibiting angiogenic factors (3, 4). One of the most important of these is VEGF, which regulates endothelial cell proliferation and vascular permeability (5).

VEGF expression is controlled in part by microenvironmental factors such as lowered oxygen pressure (hypoxia; Ref. 6) and hyperglycemia, as well as by cell-autonomous events such as oncogenic transformation (4, 7–9). There is evidence that activated ras can act synergistically with hypoxia in regulating VEGF expression, which likely explains the high levels of VEGF expression found in situ during ras-mediated tumorigenesis (10).

Previous studies (11, 12) have shown VEGF to be an important survival factor in tumorigenesis. For instance, anti-VEGF monoclonal antibodies have been shown to significantly reduce tumor growth from glioma, sarcoma, and carcinoma cell lines. These previously published studies failed to distinguish the relative contributions of tumor-cell-derived VEGF from that of adjacent cells. This is important because VEGF is also produced by tumor-associated cells like endothelial cells and macrophages, as well as by surrounding stromal cells (13). It has recently been suggested that VEGF induction in the surrounding stroma is more critical to tumor angiogenesis than VEGF production by tumor cells; this was observed in a mouse model of spontaneous mammary tumors where Fukumura et al. (13) observed that VEGF promoter activity is evident in stromal but not in tumor cells. By specifically targeting VEGF expression in tumor cells, we were able to directly address this issue.

To date, the only published genetic approach to the role of VEGF in tumorigenesis has been the use of a VEGF null ES cell line to create teratomas in immunocompromised mice. Ferrara et al. (14) reported a >10-fold decrease in tumor weight in this line relative to VEGF +/- control ES cells, with a concomitant decrease in tumor vessel density. Although these findings are informative, the teratoma is a difficult model for studying tumorigenesis for several reasons: first, it is unknown by what mechanism ES cells become tumorigenic in nude mice. Because the transforming mechanism(s) in ES cells is (are) unknown, one cannot necessarily extrapolate findings made in the teratoma model to other tumor types. In contrast, fibrosarcomas can be transformed by a defined molecular event (e.g., via activated ras transformation). This enables one to study the function of a gene within the context of a transformation pathway (15). Second, teratomas comprise not only undifferentiated cells but also several tissue types that result from extensive in vivo differentiation during tumor growth. This heterogeneity renders it virtually impossible to assay tumorigenic parameters because different cell types have varying responses to changes in levels of nutrients and oxygen. Consequently, regions of the tumor are phenotypically different in assays of vascular density and permeability as well as in cellular proliferation and apoptosis. In striking contrast, the fibrosarcoma consists of only one tumor cell type. The homogeneity of the fibrosarcoma allows accurate quantitation of VEGF-induced differences in these same assays.

The objective of the present study was to directly examine the functional importance of tumor-derived VEGF in ras transformation in vivo. To this effect, we used a strategy involving cre-loxP recombination system in ES cells (16). Through gene targeting, we generated ES cells with two conditionally targeted VEGF alleles. These cells were used to make chimeric mouse embryos, which in turn served as substrates for the isolation of transgenic EFs. We then induced the disruption of the conditional VEGF alleles in fibroblasts through cre-mediated excision of the VEGF coding sequence. Finally, we assayed the effect of the disruption on tumor formation after ras-mediated transformation of the fibroblasts. The data clearly demonstrate that tumor-derived VEGF is required for expansive tumor growth and cellular survival. The loss of tumor-specific VEGF caused a dramatic decrease in vessel density and morphology as well as a striking loss of vascular permeability.

MATERIALS AND METHODS

Construction of the VEGF Targeting Vector

Overlapping bacterial artificial chromosome clones containing the VEGF gene were obtained from Genome Systems, Ltd.; using the VEGF 165 isoform cDNA as a probe. The selectable marker included a neomycin resistance gene for positive selection fused to the HSV-1 thymidine kinase gene for negative selection. This 4.1-kb cassette was flanked by FRT sites; therefore, we could excise it from the intron after selection for targeting events (17, 18). The drug-resistance cassette (18) was cloned into the EcoRI site of intron 3. The construct was linearized and 50 µg was electroporated into R1 ES cells. After

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; ES, embryonic stem; EF, embryonic fibroblast; mEF, mouse EF; TUNEL, terminal deoxynucleotidyl transferase mediated nick end labeling.
selection under 150 μg/ml G418, clones were analyzed by XbaI digest and hybridized to a 5’ external probe, and by ApaLI digest and hybridized to a 3’ external probe. Homozygous targeted clones were obtained by selection at 6 mg/ml G418 (19).

Tissue Culture. Homozygous VEGF-targeted clones were used to generate chimeric embryos via the embryo aggregation method (20). ES cell-derived fibroblasts were selected in 400 μg/ml G418 and immobilized by stable transfection of SV40 large T antigen. Immortalized, homozygously targeted EF cells were transfected with expression vectors encoding cre recombinase (21), flaplace recombinase (18), or both. To optimize flippase expression, the transfected cells were placed in a humidified incubator at 30°C for 72 h (18). To select for cells that had lost the selectable marker, the culture was selected in 0.2 μM FIAU for 1 week. VEGF null fibroblasts were selected from the transfected pool by limiting dilution, and multiple isolates lacking exon 2 were pooled and confirmed by Southern analysis.

VEGF quantitation from cell culture-conditioned medium was performed using the Quantikine M Mouse VEGF Immunoassay Kit (R&D Systems) according to the manufacturer’s protocol. For cell growth assays, 2 × 10⁴ cells were plated on 37-mm tissue culture dishes. Cell counts were performed every 24 h in quadruplicate using a hemacytometer (Reichert). For soft agar cloning assays, 2 × 10⁴ cells were mixed in 0.4% L.M.P. Agarose (Life Technologies, Inc.) and plated onto 37-mm tissue culture dishes. Soft agar colonies were counted in triplicate after 17 days of incubation.

Generation of Fibrosarcomas
Cells (1 × 10⁵) in 100 μl DMEM were injected s.c. intrascapularly into immunocompromised mice. Tumors were harvested at 21 days postinjection for histology and weighing.

Tumor Histology
BrdU, Tunel, CD34. A subset of mice bearing fibrosarcomas (~25 grams) were injected i.p. with 500 μls of 5 mg/ml BrdU (Sigma) diluted in 0.8% NaCl, 1 mM Tris (pH 7.4), and 0.25 mM EDTA. The mice were perfused and killed 2 h postinjection, and the tumors were fixed overnight at 4°C in 4% paraformaldehyde. Tumor blocks were paraffin-embedded and cut into 10-μm sections. Sections were stained with a mouse anti-BrdU antibody (Sigma). Color was visualized by DAB-HRP assay, as described below. Sections were analyzed for apoptosis via TUNEL assay. Staining was performed with the Apoptosis Detection System, Fluorescein (Promega) as described in the manufacturer’s protocol. Apoptotic frequencies were quantitated from 104 cells were plated on 37-mm tissue culture dishes. Cell counts were performed every 24 h in quadruplicate using a hemacytometer (Reichert). For soft agar cloning assays, 2 × 10⁴ cells were mixed in 0.4% L.M.P. Agarose (Life Technologies, Inc.) and plated onto 37-mm tissue culture dishes. Soft agar colonies were counted in triplicate after 17 days of incubation.

RESULTS
Conditional Targeting of the VEGF Gene. The VEGF gene was targeted in R1 ES cells (20) resulting in the allele shown in Fig. 1. We chose to flank exon 2 by loxp sites because it encodes a portion of the export signal sequence as well as the first amino acids of the mature protein sequence. We expected that loxp-mediated excision of exon 2 would create a null allele because its loss produces a frame shift in the remainder of the coding sequence. Targeted integration of this vector into the VEGF genomic locus was detected by Southern blot analysis (data not shown). We then obtained ES cells in which both alleles were flanked by the loxp site by selecting the culture at high levels of G418. By increasing drug selection pressure, we generated homozygous targeted ES cell clones (19).

By the aggregation method (20), homozygous targeted ES cells were used to generate chimeric mouse embryos, which were then used to isolate transgenic EFs. Fibroblasts derived from the targeted ES cell chimeric embryos were selected in G418 to eliminate the non-ES cell-derived fibroblasts and then immortalized by stable transfection with SV40 large T antigen (24).

To generate VEGF cell lines in which both alleles of the gene had second exons that were flanked by loxp sites but that did not have the potentially interfering selectable cassette in the third intron, the cells were transiently transfected with flippase (pOG44; Ref. 18) to excise the selectable marker. The excision event in FIAU-resistant cells was confirmed by Southern analysis. Subsequently, the cells were transformed with a retrovirus expressing oncogenic ras (H-ras 61 L; Ref. 15). We will refer to this cell line as +/+.

To generate VEGF null (−/−) mEF cell lines, the initial SV40 large TAg-immortalized cell lines were transiently cotransfected with a flippase expression vector and a cre recombinase expression vector (pML 78; Refs. 21 and 25). This cotransfection resulted in a significant fraction of FIAU-resistant cells that had lost both the selectable cassette and VEGF exon 2. Multiple isolates of VEGF null fibroblasts
Fig. 1. Conditional disruption of the murine VEGF gene. a, a diagram of the targeting strategy designed to remove from the targeted VEGF allele: first, the FRT site-flanked neo/TK fusion cassette to produce +/− cells, and then, exon 2 of VEGF to produce −/− cells. b, Southern blot analysis of DNA digested with ApalI from +/− and −/− fibroblasts, demonstrating the loss of the loxP-flanked fragment containing VEGF exon 2. c, tumors excised from mice 21 days postinjection. d, values shown are a mean ± SE of tumor weight determined 21 days after injection (n = 6 mice). All of the tumors were well encapsulated with no sign of metastasis.

Fig. 2. Histology of VEGF +/− and VEGF −/− fibrosarcomas. All of the staining was performed on representative cross-sections of 21-day-old tumors. a and b, BrdU immunostaining (×200). Tumor-bearing mice were injected with BrdU 2 h before harvesting the tumors. Incorporation of a label was identical in the two genotypes as assayed by counting positive nuclei. c and d, apoptosis detection via the TUNEL assay (×200), with a large increase seen in TUNEL positive cells from −/− tumors. e and f, CD34 immunostaining (×400) of endothelial cells and representative vessels in paraformaldehyde-perfused tissue, showing the smaller size and number of vessels in VEGF −/− tumors.
were isolated from pooled cells by limiting dilution, and the loss of exon 2 was confirmed by Southern blot analysis (Fig. 1b). VEGF null fibroblast lines were pooled and then transformed with the (H-ras 61 L) retrovirus as described above.

To confirm that +/− cells produce normal levels of VEGF and that VEGF null cells are deficient in VEGF production, an ELISA assay was performed on conditioned media from cultured cells. There was a complete loss of detectable VEGF in conditioned media from VEGF null fibroblasts (with a detectable limit of ~3 pg/ml); +/− cultures produced ~800 pg/ml of detectable VEGF from 10^6 cells after 48 h. To control for cell-autonomous differences in ras/SV40 large T antigen-mediated transformation between the VEGF +/− and −/− cell lines, a growth curve assay and a soft agar colony formation assay were performed. In cell culture, the two cell lines proliferated at similar rates and produced similar numbers of soft agar colonies (data not shown).

Tumor Cell-Derived Expression of VEGF Is a Critical Regulator of Tumor Expansion and Neovascularization. To determine the role of VEGF in tumor growth, both VEGF +/− and −/− ras/SV40 large T antigen-transformed fibroblasts were injected into immunocompromised mice (26) to form fibrosarcomas. The tumors were then recovered, weighed, and analyzed at various times postinjection. As shown in Fig. 1, c and d, VEGF null tumors showed greatly reduced (~10-fold decrease) capacity to grow in vivo during the period assayed. Gross inspection (Fig. 1c) showed that the VEGF null tumors were not only reduced in size relative to +/− tumors but also had a less vascular appearance. These data confirm the role of tumorigenic VEGF for expansive tumor growth.

We wished to determine the cause for the decrease in tumor mass; therefore, we examined proliferation and apoptotic indices in the VEGF +/− and −/− fibrosarcomas. VEGF +/− and −/− tumors exhibit virtually identical rates of BrdU incorporation (Fig. 2, a and b), which indicates that there were no differences in cellular proliferation. However, loss of VEGF had a profound effect on apoptotic frequencies (Fig. 2, c and d). Quantitation of apoptotic indices in VEGF +/− and null tumors demonstrates that the loss of VEGF induces an 8.5-fold increase in apoptosis outside of necrotic foci. This result indicates that the loss of VEGF may inhibit tumor growth in part by affecting cell survival.

Interestingly, there was no increase in necrosis or necrotic regions in the VEGF null tumors, with the vast majority of TUNEL positive cells occurring in the more or less random distribution (Fig. 2d). In contrast, in the much larger VEGF +/− tumors, there were large regions of necrotic cells (data not shown). This may indicate that constraints on angiogenesis prevent overgrowth and the loss of vascular patency/hemorrhage, which in turn can lead to the necrosis typical of rapidly growing tumors.

Because VEGF is known to be an important angiogenic factor and because vascularization is necessary for delivery of oxygen and nutrients to growing cells, we examined the vasculature of the tumors. Immunostaining with the endothelial cell marker CD34 (Fig. 2, e and f) demonstrated that vessel density and morphology is significantly affected by the loss of tumor cell VEGF expression. Quantitation of vascular surface area over total tumor volume revealed a dramatic decrease in the proportion of the tumor volume taken up by blood vessels; the mean vascular surface area in +/− tumors was 21.4 mm^2/mm^3(SE, ±2.1) tumor volume, whereas that of VEGF null tumors was 3.6 mm^2/mm^3(SE, ±0.3) tumor volume. This represents

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Fig. 3. Vascular patency and hypoxia levels in VEGF +/− and VEGF −/− tumors. a and b, representative sections of tumors excised 1 h after the administration of the hypoxia marker EF5. EF5 staining (red) of hypoxic foci in VEGF +/− and VEGF −/− tumor sections visualized by immunohistochemical staining using a monoclonal anti-EF5 antibody. Note the increase in background EF5 binding in the VEGF −/− tumors. c and d, 10 min before the tumor was excised, Hoechst 33342 was injected into the tail vein. Diffusion of the cell-permeable tracer is indicated by blue fluorescence. Note the large amount of diffusion from VEGF +/− tumor vessels. e and f, overlay of Hoechst stain diffusion and EF5 binding to demonstrate the minimal alleviation of hypoxia by patent vessels in VEGF −/− tumors.

Fig. 4. Vascular permeability assay. Tail vein injection of a FITC-conjugated M_2 Dextran demonstrates the lack of permeability to this molecule caused by the absence of tumor produced VEGF, as evidenced by the diffuse stain in +/− tumors (a) when compared to the solely vessel-associated staining in the −/− tumor (b).
an 83% reduction in vascular surface area caused by the loss of tumor cell VEGF production. These data confirm the requirement of tumor-specific VEGF for proper neovascularization in the growing tumor.

**Loss of VEGF Increases Tumor Hypoxia, Decreases Vascular Permeability, and Alters Endothelial Structure.** Although we showed that loss of VEGF decreases vascular surface area, we were interested to know whether this decrease correlated with differences in vascular patency and oxygen levels in the tumor microenvironment.

We examined the extent of tumor hypoxia by immunodetection of the injected nitroimidazole EF5, a small molecule which forms cellular adducts under reducing conditions (27–29). Although we found hypoxic foci in both tumor types (Fig. 3, a and b), VEGF null tumors have a much higher degree of background hypoxia (as measured by EF5-dependent fluorescence, an increase of 2.42-fold by CCD pixel quantification). The increase in hypoxia is not surprising given the decrease in vascular surface area.

In the same samples, vascular patency was measured by detection of the cell-permeable fluorophore Hoechst 33342 after injection into the tail vein at a defined interval before sacrifice (30, 31). Both VEGF 

$\frac{1}{2}+/+$ and null tumors contained patent vessels (Fig. 3, c and d). Interestingly, we saw a profound increase in extravasation of the dye from patent vessels in tumors expressing VEGF. This is likely due in part to differences in luminal flow and vascular permeability. A dual filter exposure for both EF5 staining and Hoechst dye is shown in Fig. 3, e and f. In the VEGF 

$\frac{1}{2}+/+$ tumors, there is no overlap between hypoxic foci and regions of vascular diffusion. This indicates that oxygen diffusing from vessels is preventing hypoxia in the regions marked by diffusion of the Hoechst dye. In the VEGF null tumors, however, there is a reduced amelioration of hypoxia by patent vessels (Fig. 3f). The decrease in vascular diffusion of the Hoechst dye correlates with a decrease in the diffusion of oxygen into the tumor tissue. This demonstrates the dependence of the vasculature on tumor-cell VEGF expression for alleviation of tumor hypoxia.

We next investigated whether VEGF induces physiological changes in blood vessels that may further decrease the supply of oxygen and nutrients to the surrounding tumor tissue. To directly measure the effect of loss of tumor cell-derived VEGF on vascular permeability, mice with VEGF 

$\frac{1}{2}+/+$ or 

$+/+$ tumors were injected with fluorescently labeled high molecular weight dextran. As shown in Fig. 4, a and b, there is extensive diffusion of this macromolecule from the vessels into the tissue, 20 min after injection into mice with 

$\frac{1}{2}+/+$ tumors. In striking contrast, almost all of the labeled dextran remains vessel-associated in the 

$+/+$ tumors, clearly showing the essential role for tumor-derived VEGF in the increased vessel permeability that is typical of solid tumors.

The changes in vessel density and permeability in tumors lacking VEGF expression are correlated with changes in the ultrastructure of the vessels themselves. Fig. 5, A and B, show electron micrographs of representative vessels from VEGF 

$\frac{1}{2}+/+$ and 

$+/+$ tumors. The enlarged lumen and numerous fenestrations (Fig. 5B, arrows) in the VEGF 

$\frac{1}{2}+/+$ tumor are typical of the vasculature in rapidly growing solid tumors. In contrast, fenestrations were only rarely observed in VEGF null tumors. We identified two vessels with fenestrated endothelium among 200 vessels analyzed in multiple blocks from eight VEGF null tumors versus 

$>40$ of 200 vessels in eight 

$\frac{1}{2}+/+$ tumors. There were also no open interendothelial junctions detected in the VEGF null tumor vasculature examined. Furthermore, although fused clusters of caveolae, typical of VEGF-induced vessels (23, 32), are common in the neovascularature of VEGF 

$\frac{1}{2}+/+$ tumors (Fig. 5B, inset), none were detected in the VEGF null tumors ( 

$>200$ vessels examined from 8 null tumors). Caveolae have also been called vesiculo-vacuolar organelles and are thought to contribute to the hyper-permeability often observed in tumorigenic vasculature (33). These findings definitively establish the requirement for tumorogenic VEGF expression for structural alterations of the vasculature leading to increased permeability during tumorigenesis.

**DISCUSSION**

We have developed a novel system for studying tumor angiogenesis by generating ras/SV40 large T antigen-transformed fibroblasts that do not express VEGF, and we have defined a baseline phenotype for VEGF null tumors. We have provided direct and definitive evidence that the loss of tumorogenic VEGF causes dramatic decreases in tumor size, vascular density, and permeability. These data not only confirm previously published data from VEGF blocking studies but also demonstrate that tumor-derived VEGF is sufficient to induce these changes. This model system will allow other angiogenic factors to be measured for their ability to sustain tumor growth by introducing them into a VEGF null background.

In contrast to the data reported by Fukumura et al. (13), our model demonstrates a dependence on tumorogenic expression of VEGF for solid tumor growth. Thus, although stromal cell production of VEGF may play a significant role in tumor angiogenesis, it is clearly not sufficient in our model to rescue tumor growth and vascularization. There is an implicit connection between transformation by activated ras and angiogenesis: activated ras acts to induce VEGF expression and synergistically enhance its up-regulation by hypoxia. Our work shows that recombine-mediated deletion of the VEGF gene can be used to study the role of this growth factor from its establishment to late-stage growth of an injected tumor cell line.

Tumors undergoing rapid expansion experience changes in their microenvironments as they expand, including depletion of oxygen (34), increase in acidification (35), and disruption of the extracellular matrix. The response to these stimuli likely determines the degree of success of tumorogenic outgrowth and, ultimately, metastasis. Comparison of tumors in which the tumor cells either express VEGF, or cannot, reveals that VEGF likely regulates tumor oxygenation by stimulating blood vessel density and vascular permeability. The surface area of blood vessels per unit volume of tumor was six times higher in tumors that expressed wild-type levels of VEGF than in null tumors; thus each tumor cell is on average much closer to a vessel in wild-type tumors. We have shown that the absence of tumor-specific expression of VEGF reduces vessel surface density and thereby decreases the area of exchange between the vessel lumen and the tumor’s interstitial fluid. We have also shown a complete loss of VEGF 

$+/+$ tumor’s permeability to a labeled dextran when compared with the typical and large-scale “leakiness” of VEGF 

$\frac{1}{2}+/+$ tumors.

Hallmarks of tumor neovascularature are the fenestrations and caveolae found in the endothelium seen in the 

$\frac{1}{2}+/+$ tumor in Fig. 5b. Since the vascular structure of the tumor derives from the host animal and not the tumor, its potential for expression of VEGF is unaltered. However, it is clearly dependent on VEGF produced by the tumor cells for the induction of fenestrations and caveolae, which do not arise in the vessels of VEGF null tumors. Fenestrations are correlated with increased permeability of the vasculature (32), and the caveolae are associated with the induction of fenestrations (36). This model system has for the first time definitively shown that these structures are dependent on VEGF expression by transformed cells for their occurrence in a particular tumor type.

Folkman and colleagues (3, 37–39) have shown that anti-angiogenic stimuli inhibit tumor growth through increased apoptosis in the absence of alterations in tumor cell growth rates. We see clear indication of the same phenomenon here. Two properties of the tumorogenic vasculature have been changed by the lack of VEGF expression,
that of neovascular density and structure/permeability. The relative contribution of each of these to the large increase in apoptosis is not yet clear. Both may play an important role and act jointly to alleviate hypoxia.

There is evidence that other angiogenic factors may also be required for tumorigenesis and may partially rescue tumorigenesis in the absence of VEGF. As anti-VEGF therapy becomes a realistic approach to treat cancer patients, it is crucial to predict what factor(s) may substitute for the absence of VEGF. Our VEGF null model system allows us to directly answer this question. For example, by introducing a basic fibroblast growth factor (FGF2) expression construct into VEGF null, ras-transformed fibroblasts, we can directly measure the degree of functional redundancy with VEGF as measured by tumorigenic rescue. In addition, it may be interesting to allow VEGF null tumors to revert to a tumorigenic phenotype through consecutive passages of the cells in immunocompromised mice. This approach may select for angiogenic factors that will rescue tumorigenesis in vivo.

A VEGF null background is also useful for dissecting the roles of the separate VEGF splice variants. Our system would allow the reintroduction of these isoforms under the control of a strong promoter, to measure the function and relative importance of each isoform in tumorigenesis. It has been postulated that the differences in solubility between the isoforms result in a difference in bioavailability in the tumor environment. However, it has not yet been possible to test whether the splice variants actually have different biological functions. Although this is a subject of a future publication, we have recently generated cell lines which express single VEGF splice variants, and we find that each isoform can at least partially restore the VEGF null phenotype (data not shown). Considering that VEGF functions as a mitogen, a permeability agent, and a chemotactic factor, it is certainly plausible that the splice variants contribute separately to these diverse functions. In fact, it has recently been reported that VEGF splice variants differentially bind to a novel VEGF receptor, neuropilin-1, which is highly expressed by tumor cells (40). A VEGF null background would be ideal to assay the relative importance of the receptor-ligand interaction of each VEGF splice variant with this novel receptor. Finally, though we have demonstrated that VEGF is required for ras/SV40 large T antigen-mediated tumorigenesis, it would be interesting to know whether other transforming oncogenes

Fig. 5. Electron micrograph of neovasculature from VEGF +/+ and VEGF −/− tumor. A, VEGF −/− tumor vessel with tight interendothelial junction, characteristic of normal but not tumor vessels. There is also an absence of fused endothelial caveolae. Endothelial cell fenestrations, a hallmark of tumor vasculature, are also absent. B, VEGF +/+/f tumor vessel with fenestrated endothelium (fenestrae, arrowheads) with open or loose interendothelial junctions (arrows). VEGF +/+/tumor vessels generally had increased luminal area, and endothelium was attenuated compared with VEGF −/− tumor vessels. The endothelial morphologies observed in the VEGF +/+/tumors are characteristic of VEGF-induced neovascularite. B, inset, vessel from VEGF +/+/ tumor demonstrating fused and clustered caveolae, also characteristic of VEGF-induced neovascularite. Bars, 500 μm.
also require VEGF for tumorigenesis. This approach may help to elucidate novel angiogenic pathways which are triggered by separate oncogenes. The results described in this paper demonstrate a requirement for tumor cell expression of VEGF for expansion of the tumor mass, and an illustrate an approach for evaluating the role of angiogenesis and angiogenic factors in tumor growth. The lack of typical tumor endothelial cell morphology and function in VEGF−/− tumors clearly demonstrates that tumorigenic VEGF is a primary mediator of changes in endothelial cell structure, vessel density and permeability during ras/SV40 large T antigen-mediated tumorigenesis.

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