Neovascularization Induced by Vascular Endothelial Growth Factor Is Fenestrated

W. Gregory Roberts and George E. Palade

Cellular and Molecular Medicine, University of California-San Diego, La Jolla, California 92093-0651

ABSTRACT

We have reported previously that topical administration of vascular endothelial growth factor165 (VEGF) to a microvascular bed supplied with a continuous endothelium can rapidly induce the formation of endothelial fenestrations (W. G. Roberts and G. E. Palade, J. Cell Sci., 108: 2369–2379, 1995). From these results, we hypothesized that tumor vasculature, in general, may also be fenestrated because it has been reported that tumor secretion of VEGF causes the surrounding host vasculature to invade and feed the growing tumor. Using electron microscopy to characterize the endothelial cell morphology in tumor vessels from either the periphery or the core of the tumor and immunoblotting to detect secreted VEGF, we analyzed the vasculature of human and murine neoplastic tumors grown s.c. in male nude mice. To clarify the role of VEGF165, two models were used: (a) Chinese hamster ovary (CHO) cells stably transfected with hu VEGF165 and injected into mice (VEGF:CHO tumors); and (b) slow-release pellets containing purified VEGF or basic fibroblast growth factor implanted on the rat cremaster muscle. All tumors had vessels with fenestrated endothelium, open interendothelial junctions, and clustered fused caveolae. From all of the peripheral tumor vessels observed, fenestrated endothelium was observed in 41% from EMT, 35% from MIS, 37% from U87, and 56% from VEGF:CHO tumors, whereas surrounding skin and muscle, from which tumor vessels were derived, had fenestrated endothelium in 2% and 0% of all vessels, respectively. Additionally, further analysis revealed a substantial decrease in the anionic glycoscalyx on the luminal face of the fenestral diaphragms in endothelium from tumors (especially VEGF:CHO) when compared to intestine or pancreas. Because the host tissue microvascular endothelium which supplies the tumor is not fenestrated, tumors can transform nonproliferating, nonfenestrated vessels into proliferating vessels, many of which have fenestrated endothelium. These data provide evidence that chronic VEGF exposure can induce fenestrations in nonfenestrated endothelium similar to the fenestrated endothelium found in tumor vessels.

INTRODUCTION

Tumor growth is angiogenesis dependent, which means that they necessitate the ingrowth of a vascular supply from the surrounding tissues to proliferate and metastasize (1–3). Tumor vasculature is characterized by its tortuous architecture (4), irregular blood flow (5, 6), and increased permeability relative to normal vessels (7). Because tumor vascular morphology directly impacts its functional hemodynamics and permeability, it is critical to understand how angiogenic growth factors, responsible for the neovascularization, affect tumor vascular morphology. Presently, there are discrepancies between tumor vascular physiologists and morphologists regarding the mechanisms and structures responsible for increased vascular permeability (8, 9).

Most, if not all, tumors tested secrete an endothelial cell-specific mitogen, known as VEGF3 (10). Also known as vascular permeability factor, it was originally described as a tumor-secreted protein that significantly increased vascular permeability (11). Alternative splicing of the VEGF gene transcript accounts for four isoforms of 121, 165, 189, and 206 amino acids, of which the 165-amino acid form is believed to be the most abundant in vivo (12). However, using RNase protection, all three shorter isoform mRNAs were found in rat brain, kidney, lung, spleen, and heart; the 165-amino acid isoform was not always the most abundant (13). At present, it is not known whether there are differences in mitogenic or permeability potency among the isoforms. VEGF165 is an N-glycosylated protein most commonly found as a homodimer of Mr ~46,000.

VEGF is produced by many cell types other than tumors, including folliculocystic cells, keratinocytes, macrophages, and possibly podocytes of renal glomeruli among others (10, 14–16). VEGF is likely responsible for angiogenesis in wound healing and a variety of pathologies, including solid tumors, diabetic retinopathy, psoriasis, and rheumatoid arthritis (17–20), as well as vasculogenesis in embryos (21–23). In fact, the loss of a single allele of VEGF165 was lethal to the mouse embryo by day 12, thus emphasizing the critical role of this growth factor in the normal development of the vascular system (24, 25). VEGF expression is tightly regulated to times of blood vessel growth and is up-regulated by hypoxia (26–28). However, persistent expression by epithelial cells adjacent to fenestrated endothelium in the adult has led to the hypothesis that VEGF is involved in the induction and/or maintenance of fenestrae in endothelia (21). More recently, it was shown that VEGF directly and rapidly induces the formation of fenestrations and the swelling, clustering, and fusion of plasmalemmal vesicles in nonfenestrated endothelium of the skeletal muscle and skin (29).

There are two related high affinity receptors known for VEGF, fms-like tyrosine kinase (Flt-1) and fetal liver kinase (Flk-1), the expression of which appears to be limited to the endothelium (30, 31). The receptors are present on tumor vessels and up-regulated on neovascularization in pathologies and normal development (27, 32). Similar to VEGF expression, Flk-1 and Flt-1 are closely regulated to times of vasculogenesis and angiogenesis (23, 28). At present, it is realized that VEGF and its receptors are critical factors in tumor angiogenesis and metastasis (33).

In this report, we demonstrate that: (a) every tumor tested had fenestrated endothelium with dilated, irregular, coalescing plasmalemmal vesicles, and open interendothelial junctions; (b) on these accounts, tumor vasculature is clearly different from the host tissue microvasculature supplying the tumor; and (c) the fenestral diaphragms in tumor neovasculature have significantly less anionic charge than in normal fenestrated endothelia. Evidence obtained on tumor models and slow-release pellets clearly shows that: (a) VEGF alone can induce fenestrated endothelium, open interendothelial junctions, and modify plasmalemmal vesicles, morphologies commonly associated with tumor microvasculature; (b) chronic administration of VEGF in low doses can induce fenestrations without a visually detectable angiogenic response; and (c) our findings provide morpho-

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2 To whom requests for reprints should be addressed, at Cellular and Molecular Medicine-0651, University of California-San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0651. Phone: (619) 534-7712; Fax: (619) 534-8549.

3 The abbreviations used are: VEGF, vascular endothelial growth factor; hu rVEGF165, human recombinant VEGF isoform 165; CHO, Chinese hamster ovary; bFGF, basic fibroblast growth factor; CF, cationic ferritin.

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MATERIALS AND METHODS

VEGF-transfected Cells. cDNA encoding human VEGF<sub>165</sub> was cloned into the pcDNA3neo mammalian expression vector. pcDNA3neo constructs were stably transfected into CHO cells. Following clonal selection for high levels of VEGF secretion, the VEGF-CHO cells were injected (5x10<sup>4</sup>-1x10<sup>6</sup> cells) s.c. on the flank of male nude mice as described below.

Tumor Growth. Male BALB/c, nude mice and DBA/2 mice (25-30 g) were used throughout the study. We examined three tumors: EMT, a mammary carcinoma syngeneic for BALB/c; M1S, a rhabdomyosarcoma syngeneic for DBA/2 mice; and U87, a human grade IV glioblastoma. These tumors were maintained in vivo by s.c. injection on the flank of nude mice. Because in vivo passaging naturally selects for aggressive, faster growing, and more angiogenic tumors, all studies used tumors that had been passed at least seven times in vivo. The only exception was tumors generated with VEGF-transfected CHO cells that were maintained in culture under 0418 selection until s.c. injection. All procedures were in accordance with NIH standards and were reviewed by an institutional animal care committee.

VEGF and bFGF Pellets. hu rVEGF<sub>165</sub> was purified and quantified as described previously (29). bFGF was purchased from R&D Systems (Minneapolis, MN). Preparation of growth factor-containing Elvax pellets was described previously (34). Briefly, Elvax 40P pellets (DuPont, Wilmington, DE) were washed exhaustively with numerous changes of 100% ethanol. After drying, they were dissolved in methylene chloride to make a 20% w/v solution. Lyophilized growth factors were made in the scrotum, and a pellet (usually 1-3 mg; 10-200 ng of VEGF, 0.5-2 μg of bFGF) was placed on top of the cremaster muscle at least 1 cm from the incision, which was sutured with 3-0 prolene. After 10-20 days, the cremaster was excised with pellet in place. Some animals received an i.v. injection of carbon black (0.5 ml/kg) prior to tissue removal (29). Animals were anesthetized, and tissues were fixed in situ for 10 min with 5% freshly prepared formaldehyde prior to removal, trimming, and routine processing, as described below. Analysis was completed using 30 samples taken from 12 rats for both VEGF and bFGF pellet studies.

Electron Microscopy. Sample preparation was reported previously (29). Tumor specimens were taken from the core or periphery (including incoming vessels just outside of the tumor border) of 5-10 mm diameter tumors with no visible signs of necrosis using one of the following protocols: (a) 3% freshly prepared formaldehyde and 1.5% glutaraldehyde was injected s.c. around the tumor, marking the skin to ensure the injection did not disrupt the peripheral vessels prior to trimming; (b) whole tumors were removed, immersed, and trimmed in 3% formaldehyde; or (c) unfixed tumors were removed and trimmed into small pieces and immediately fixed by immersion in 1.5% glutaraldehyde. Regardless of the removal and trimming procedure, all trimmed pieces were further fixed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate-HCl (pH 7.4) with 5% sucrose for 1 h. Following three washes with 0.1 M cacodylate buffer with 7.5% sucrose, specimens were postfix in cacodylate-buffered (pH 7.4) 1% OsO<sub>4</sub> on ice for 1 h, en bloc stained with uranyl acetate for 1 h, dehydrated, embedded in Epon 812, and cured for 18-24 h at 60°C. Thin (30-55 nm) sections were cut (Reichert-Jung Ultracut E; Vienna, Austria), picked up on nickel grids, and stained with uranyl acetate and lead citrate before examination and photographing on a Phillips CM10 electron microscope at 80 kV. Morphological analysis on open interendothelial junctions and endothelial fenestrations was completed on non-injected mice. There was no discernable difference in the results among the different tumor removal and trimming processes described above. Data were compiled from at least three blocks from no less than 10 tumors of each type. Many grids from each block containing on average of four sections were used (minimum of 360 sections from both peripheral and core vessels).

For studies using CF (Sigma Chemical Co., St. Louis, MO), tumor/VEGF:CHO-bearing animals were sacrificed 3-5 min following an i.v. injection of 0.2 ml of CF (70 mg/kg; Ref. 29); tissue samples were then processed for electron microscopic observation. Analysis was completed using 18 tumor, 12 pancreatic, and 12 intestinal samples taken from six mice injected with CF.

Immunoblotting. Tumors were aseptically removed and immediately homogenized in PBS supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, aprotonin (0.2 μTIU/ml), and 1 mM iodoacetamide (100 mg of tissue/ml buffer), then passed through a 25-gauge needle three times, and centrifuged at 1000 RPM for 5 min; the clarified lysate was precipitated with heparin-Sepharose (Pharmacia Biotech, Inc., Uppsala, Sweden) for 1 h at 4°C. Following washing in buffer, the beads were boiled in SDS-sample buffer, and proteins were separated in a 12% acrylamide gel (acrylamide; National Diagnostics, Atlanta, GA; other chemicals were from Bio-Rad, Richmond, CA), transferred to polyvinylidene difluoride or nitrocellulose membranes, blocked with 5% milk/0.1% Tween 20 in Tris-buffere saline (T-TBS; room temperature, 1 h), incubated for 1 h at room temperature in 1 μg/ml with polyclonal anti-VEGF Ab (Santa Cruz Biotech, Santa Cruz, CA or Peprotech, Rocky Hill, NJ), washed with 1% milk in T-TBS (three times for 10 min each time), incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit Ab (Biodesign International, Kennebunk, ME), washed, and detected by enhanced chemiluminescence (Pierce). Band densities were determined from single membranes containing all samples using a densitometer. Results are representative of more than 10 blots using, on average, 15 tumors of each type.

Statistics. For the percentage of fenestrated vessels, hypergeometric (exact) tests and χ<sup>2</sup> tests on skin versus tumors and muscle versus tumors were used. For the number of fenestrations per vessel, hypergeometric tests and Wilcoxon tests were used. For CF results, χ<sup>2</sup> tests on percentage of CF labeling of intestine or pancreas versus each tumor were used. For the percentage of fenestrated vessels in control versus VEGF or bFGF pellets, χ<sup>2</sup> tests were used.

RESULTS

Neoplastic Tumors

Results described in this section refer to the murine mammary tumor, EMT, murine rhabdomyosarcoma, M1S, and the human glioblastoma, U87, growing in either the syngeneic host (EMT or M1S) or in nude mice. All tumors were 8-10 mm in diameter and had no obvious necrosis. The vast majority of vessels growing into these s.c. tumors originated from the underlying muscle. Moreover, all tumors had some vessels growing through the s.c. space the entire length of the animal, coming from as far away as the axillary region (4-5 cm away). These vessels are easily seen because of their size through the relatively transparent skin of the nude mouse.

Vessels: Differentiation between the Tumor Core and the Periphery

Core Vessels. Because vessels in the tumor core were tortuous with abnormal lumen sizes and shapes, they were most often recognized because of RBCs in their lumens (Fig. 1). Regardless of the host (syngeneic or nude) or tumor type, these vessels had common features: open interendothelial junctions, fenestrated endothelium, and abnormally convoluted lumina. They were obviously abnormal and pathological with a loss of the normal perselective barrier, evidenced by large interendothelial gaps and absent or discontinuous basement membrane. Many core vessels had more than one open interendothelial junction each, some measuring up to ~700 nm (Fig. 1, *) which allowed plasma proteins and cell debris access to the tumor cell interstitium. Additionally, endothelial blebbing into the lumen was common.

Peripheral Vessels (within 2 mm of the Tumor Border). Common among peripheral vessels, regardless of tumor type or mouse strain, were large “venular-like” vessels with highly attenuated endothelium (Fig. 2). Occasionally, neovascularule resembling arterioles with a prominent internal elastic lamina and
In both core and peripheral vasculature, the endothelium was active in protein synthesis, demonstrated by extensive electron dense rough endoplasmic reticulum and numerous polysomes (Figs. 1 and 2). Whereas open endothelial gaps were the most common abnormality in core vessels, peripheral vascular endothelium was characterized by fenestrations and attenuation. Fenestrated endothelium appeared in larger peripheral vessels, as well as small, capillary-sized vessels, but not in the arteriolar vessels. Fenestrations were morphologically identical to normally occurring diaphragmed fenestrations of approximately 60 nm in diameter with a central knob and radiating fibrils (Figs. 1 and 2, arrows and arrowheads).

Another feature noticed preferentially in peripheral vessels were clusters of swollen (3–4 times normal), deformed (irregular and no longer spherical), and fused caveolae (Fig. 2, inset). These structures were evident in all tumors but did not appear as widespread as fenestrations, especially in the peripheral vessels. Noticeably, between the fused vesicles were fenestral diaphragms cut in cross-section (Fig. 2, inset, arrowheads) and en face (Fig. 2, inset, arrows).

Recent work has shown that all of these changes are elicited by VEGF upon topical application to vascular beds provided with a continuous (nonfenestrated) endothelium. Therefore, it is hypothesized that the changes described are the response of ingrowing microvasculature to the VEGF secreted by the tumors. Two obvious questions present themselves: (a) can an experimental model using nontumor cells producing VEGF upon transfection elicit comparable vascular morphologies as the tumors? and (b) do these tumors produce VEGF?

**VEGF-transfected CHO Cell Tumors**

To determine whether transformed tumor cells were necessary to generate neovascularization with endothelial fenestrations and open junctions, we used CHO cells stably transfected with hu rVEGF<sub>165</sub>. The VEGF:CHO cells, unlike vector-alone transfected cells, grow into well-vascularized “tumors” in ~70–80% of injected nude mice. Significantly, these VEGF:CHO tumors also had vessels with fenestrated endothelium and open junctions (Fig. 3). The differences in vascular...
morphology between peripheral and core vessels were less pronounced in the VEGF:CHO tumors than in neoplastic tumors. Although present, open junctions were not as common and were smaller in the core vessels of VEGF:CHO tumors compared to M1S, EMT, or U87 tumors in nude mice (compare Figs. 3 and 2, *). In comparison to the core vessels of the other three tumors, VEGF:CHO core vessels were more similar to peripheral tumor vessels. They had considerably larger lumina and were easily identifiable and not as convoluted. Interestingly, in VEGF:CHO tumor vessels, the basement membrane appears less disrupted than in neoplastic tumor vasculature, emphasizing that many factors influence the ultimate tumor vascular morphology. Similar results were obtained when baby hamster kidney cells stably transfected with VEGF:PNUT constructs were injected s.c. into nude mice (data not shown).

VEGF165 was detectable in lysates and was secreted by all four tumor types (Fig. 4). Compared to the other three tumors, VEGF:CHO tumors had about six times more VEGF in the lysates. VEGF from all tumor types (Fig. 4). Compared to the other three tumors, VEGF:CHO tumors had about six times more VEGF in the lysates. VEGF from all tumor types (Fig. 4). Compared to the other three tumors, VEGF:CHO tumors had about six times more VEGF in the lysates. VEGF from all tumor types (Fig. 4). Compared to the other three tumors, VEGF:CHO tumors had about six times more VEGF in the lysates. VEGF from all tumor types (Fig. 4). Compared to the other three tumors, VEGF:CHO tumors had about six times more VEGF in the lysates. VEGF from all tumor types (Fig. 4). Compared to the other three tumors, VEGF:CHO tumors had about six times more VEGF in the lysates. VEGF from all tumor types (Fig. 4). Compared to the other three tumors, VEGF:CHO tumors had about six times more VEGF in the lysates. VEGF from all tumor types (Fig. 4). Compared to the other three tumors, VEGF:CHO tumors had about six times more VEGF in the lysates. VEGF from all tumor types (Fig. 4). Compared to the other three tumors, VEGF:CHO tumors had about six times more VEGF in the lysates. VEGF from all tumor types (Fig. 4). Compared to the other three tumors, VEGF:CHO tumors had about six times more VEGF in the lysates. VEGF from all tumor types (Fig. 4).

Although vector-alone transfected CHO cells did not generate tumors in nude mice, to eliminate the possibility that CHO cells may contribute other factors that modify the vasculature, the system was further simplified by using purified growth factors in slow-release pellets.

Growth Factor Slow Release Pellets: VEGF and bFGF

To avoid any influences from cellular secretions and to test the influence of specific angiogenic growth factors alone, either hu rVEGF165 or bFGF (hu recombinant or bovine) were incorporated into slow release pellets. Doses of 10–200 ng of VEGF and 0.5–2 µg of bFGF per pellet were implanted on rat cremasters and left in place for 10–20 days. VEGF pellets with 50 ng or higher generated a visible ingrowth of vessels. Similar to the tumors, VEGF-induced neovascularization had open junctions and fenestrated endothelium (Fig. 5a). Elvax pellets with 10 ng of VEGF did not induce new vessel growth. However, there was evidence of carbon black leak, indicative of opened interendothelial junctions. Additionally, vessels with extensively fenestrated endothelium could be found (Fig. 5a), although not as frequently as in tumors. Recalling that these pellets were in place for 10 to 20 days, these results provide evidence that VEGF can induce and maintain a fenestrated endothelium without an angiogenic response. No similar modifications were found in control pellet implanted plants. Surprisingly, bFGF-induced neovascularization also had open junctions (Fig. 5b, *) and fenestrations in the endothelium (Fig. 5b, inset, arrowheads). Non-angiogenic doses of bFGF were not tested.

Fenestral Diaphragms

To further elucidate possible differences between fenestrations in tumor neovascularization and those occurring normally in adult fenestrated endothelium, the ability of CF to label fenestral diaphragms was studied. Normal fenestral diaphragms have an anionic glycocalyx (recognized by CF) on their luminal surface, which is reported to add to their permeability. Adult small intestine and pancreas are supplied with fenestrated endothelium and were used as positive controls. When bound to the fenestral diaphragm, CF forms a ball with discreet ferritin particles (Fig. 6, a–d, arrowheads). In some cases, CF was able to pass through the diaphragm and localize in the extravascular space (Fig. 6d, >>). Not quantitated was the amount of CF bound to the fenestral diaphragm, which was always greater in control compared to tumor vessels (Fig. 6, compare a and b to c and d). The percentage of fenestral diaphragms labeled with CF in intestine, pancreas, and tumors is shown in Fig. 6e. Whereas 77–91% of all fenestral diaphragms in pancreas and intestine, respectively, are labeled with CF, only 30–50% of all diaphragms in tumor neovascularization are labeled. More striking is the fact that only 7% (20 of 284) of all fenestral diaphragms in neovascularization of VEGF:CHO tumors were labeled with CF. These data demonstrate substantial differences in the surface charge characteristics of fenestral diaphragms in neovascular endothelium compared to adult fenestrated endothelium.

![Diagram](Fig. 4. Tumor-secreted VEGF/Western blot. Tumor lysates (equivalent protein) were incubated with heparin-Sepharose, washed, and boiled in SDS loading buffer; proteins were separated by SDS-PAGE (12%). Following transfer to nitrocellulose, VEGF was detected using affinity-purified polyclonal anti-VEGF. VEGF:CHO tumors secrete about six times more VEGF compared to other tumors as determined by densitometric analysis. Data are representative of numerous experiments. Left: molecular weight markers.)

![Diagram](Fig. 5. a, VEGF Elvax pellets. VEGF pellets (10–200 ng) were implanted on the rat cremaster for 10–20 days. At doses above 25 ng, VEGF induced substantial vessel growth, some of which had fenestrated endothelium (inset, upper right, 100 ng of VEGF). At doses of 10 ng of VEGF per pellet, pellets did not induce visible angiogenesis but did cause increased vascular permeability via open interendothelial junctions, as demonstrated by carbon black extravasation (inset, upper left) and induced and maintained fenestrations (arrowheads) in endothelium, which normally do not have fenestrations. Control pellets did not induce vascular permeability, vessel growth, or fenestrated endothelium. Bars: main micrograph, 200 nm; insets: upper left, 200 nm; upper right, 100 nm. b, bFGF Elvax pellets. Pellets containing bFGF (1–3 µg) were angiogenic. Levels of bFGF that did not induce vessel growth were not tested. Angiogenic vessels commonly had open junctions (*), which allowed extravasation of carbon black. Interestingly, some bFGF-induced neovascularization also had fenestrated endothelium (inset, lower right). Bars, 300 nm.)
FENESTRATED VESSELS IN VEGF AND TUMOR NEOVASCULATURE

Morphometry

Because micrographs suggested consistent variations and because in our findings fenestrations represent the dominant phenotype of peripheral tumor vessels, a morphometric survey was carried out to quantitatively document the findings and to show that the fenestrations are not occasional but are common features of the endothelium of tumor and tumor model vasculature (Fig. 7). Greater than 34% of all the vessels observed in the neoplastic tumor periphery had fenestrated endothelium, whereas open junctions were noticeable in 3.5–7% of these vessels (Fig. 7a). When compared to the percentage of normal vessels (from which tumor vessels are derived) with fenestrated endothelium, 2.4% for skin (3 of 160) and 0% for muscle (0 of 251), these differences are striking. Moreover, the extent to which VEGF may be responsible is made more obvious by the fact that 55.5% of all peripheral vessels observed in VEGF-CHO tumors had fenestrated endothelium, whereas open junctions were noticeable in all the vessels observed in the neoplastic tumor periphery had fenestrated endothelium. Significantly, VEGF:CHO tumors have the greatest percentage of vessels with fenestrated endothelium. Control and growth factor pellets placed on the cremaster are shown to the right. Although both bFGF and VEGF induced neovasculature with fenestrated endothelium and open junctions, they are not significantly different. *, all tumors versus skin or muscle, P < 0.001; both VEGF and bFGF pellets versus control, P < 0.001 (χ² test). b. Fenestrations per vessel profile. Fenestrated vessels from the same samples as described in a were also characterized as to the number of fenestrations per vessel profile. The number of fenestrations per vessel profile in all tumors is significantly greater than normal skin and muscle but not different among tumors. However, significant differences can be seen between the VEGF and bFGF pellets. Although both induce fenestrated neovascularization, the extent of this effect is much greater with VEGF. Bars represent means with SE. *, all tumors versus skin, P < 0.025 (Wilcoxon test).

Fig. 7. a, fenestrations and open junctions. Peripheral vessels containing endothelium with fenestrations or open junctions in normal skin and muscle, tumors, or pellets were counted, and the percentage of the total number counted (n) are displayed. Normal skin and muscle refer to skin overlying and skeletal muscle underlying tumors growing on the flank of nude mice. Tumor vessels are derived from vessels originating in these tissues. There is a noticeable increase in the percentage of tumor vessels that have fenestrated endothelium and open junctions compared to the normal tissues. Significantly, VEGF:CHO tumors have the greatest percentage of vessels with fenestrated endothelium. Control and growth factor pellets placed on the cremaster are shown to the right. Although both bFGF and VEGF induced neovasculature with fenestrated endothelium and open junctions, they are not significantly different. *, all tumors versus skin or muscle, P < 0.001; both VEGF and bFGF pellets versus control, P < 0.001 (χ² test). b. Fenestrations per vessel profile. Fenestrated vessels from the same samples as described in a were also characterized as to the number of fenestrations per vessel profile. The number of fenestrations per vessel profile in all tumors is significantly greater than normal skin and muscle but not different among tumors. However, significant differences can be seen between the VEGF and bFGF pellets. Although both induce fenestrated neovascularization, the extent of this effect is much greater with VEGF. Bars represent means with SE. *, all tumors versus skin, P < 0.025 (Wilcoxon test).
FENESTRATED VESSELS IN VEGF AND TUMOR NEOVASCULARITY

Table 1  Fenestrations per peripheral vessel profile

<table>
<thead>
<tr>
<th>No. of vessels</th>
<th>Range</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>124 (2.4)</td>
<td>1–2</td>
</tr>
<tr>
<td>Normal</td>
<td>250 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>88 (41)</td>
<td>1–54</td>
</tr>
<tr>
<td>EMT-6</td>
<td>181 (35)</td>
<td>1–36</td>
</tr>
<tr>
<td>Murine mammary carcinoma</td>
<td>62 (37)</td>
<td>1–44</td>
</tr>
<tr>
<td>M18</td>
<td>119 (56)</td>
<td>1–35</td>
</tr>
<tr>
<td>Murine rhabdomyosarcoma U87</td>
<td>133 (0.8)</td>
<td>—</td>
</tr>
<tr>
<td>Human glioblastoma</td>
<td>84 (8.3)</td>
<td>3–52</td>
</tr>
<tr>
<td>VEGF-CHO</td>
<td>112 (6.3)</td>
<td>1–14</td>
</tr>
</tbody>
</table>

a Number of vessels counted (percentage of fenestrated vessels). Numbers in parentheses, percentage. 
b Range of fenestrations per vessel profile. 
c Average number of fenestrations per vessel profile.

It must be emphasized that the vessels growing into these tumors are derived from the surrounding normal tissues (i.e., skeletal muscle and skin), which rarely, if ever, have fenestrated endothelium or open interendothelial junctions (Table 1; Refs. 47 and 48).

It is also important to emphasize that the neoplastic tumors used in this study were continually grown in vivo. In our experience, tumors generated from cells maintained in vitro grow much slower, produce less VEGF, and generate fewer vessels with less fenestrated endothelium compared to tumor cells maintained in vivo. Our method of in vivo maintenance was utilized so that tumors were naturally selected to be more aggressive and angiogenic.

Tumor vasculature has long been characterized as hyperpermeable (49–51). However, there is some controversy regarding the mechanisms and structures responsible for the increased permeability of tumor vessels. Physiological studies have demonstrated that latex beads and sterically stabilized liposomes as large as 600 nm in diameter were able to extravasate into the extravascular space after i.v. injection (9, 52), suggesting large open interendothelial junctions and an incomplete or absent basement membrane in tumor vessels. Other studies suggest that tumor vessel hyperpermeability is primarily the result of transport through fused clustered caveolae often found in tumor vasculature, referred to as vesiculo-vacuolar organelles (8, 53, 54). However, the limiting parameter in the vesiculo-vacuolar organelles is the area of fusion between enlarged vesicles and plasma membrane, rarely larger than 70 nm and often provided with fenestrating diaphragms (8, 53). Therefore, the larger tracers could only extravasate through an open endothelial junction, consistent with our findings.

Recently, it has been suggested that open endothelial gaps in hyperpermeable venules are artifactual results due to the colloidal tracers used in the studies (54). In the present study, all of the morphological data on tumor endothelial open junctions/gaps and fenestrations (Fig. 6; Table 1) were obtained on non-injected animals; therefore, the results were not due to exogenous tracers. In principle, the open endothelial gaps observed in the tumor vasculature may be transcellular as well as intercellular. However, we do not have data to support the first possibility. In fact, from the earliest observations (55, 56) to more recent studies with better resolution (57), the findings demonstrate that increased endothelial permeability due to inflammatory mediators is the result of open interendothelial junctions.

Can Fenestrations Observed in Tumor Vessels Account (in Part) for the Increased Permeability? Naturally occurring fenestrated endothelium is readily permeable to water and small solutes (8, 53, 58, 59), but permeability to albumin does not appear to be substantially greater in fenestrated endothelium compared to continuous endothelium (60). The permeability through fenestrae should be much greater because the openings between the radiating fibrils average 6 nm (60, 61). In fact, the glyocalyx and basement membrane contribute more to the permeselectivity than the physical structure of the fenestral diaphragm alone (60), such that the luminal glyocalyx on capillary endothelium actually decreases vascular permeability (62–64). Normal fenestral diaphragms have a unique concentration of anionic sites recognized by CF (65, 66), which undoubtedly contribute to their permeselectivity. If the glyocalyx and the basement membrane are decreased or absent in fenestrated neovascular endothelium, the actual permeability of fenestrated neovascular endothelium should be much greater than in naturally occurring fenestrated endothelium. It is already known that the basement membrane is absent and/or discontinuous in tumor vessels (67). Our results also indicate that fenestral diaphragms in tumor neovascular endothelium have a significantly decreased anionic glyocalyx on their luminal surface compared to adult fenestrated endothelium. Additionally, when fenestrations in tumor vessel endothelium are labeled, they
appear to bind less CF compared to fenestral diaphragms in pancreas and intestine, which may also be representative of a decreased anionic charge on diaphragms in tumor vascular endothelium. Therefore, the fenestrated neovascular endothelium is likely to be more permeable to larger solutes like albumin (68–70). However, the exact mechanisms by which VEGF may modify the glyocalyx of fenestral diaphragms in neovascular tissue are, as yet, unclear.

Can Model Systems Clarify the Role of VEGF? Tumors inherently generate a complex environment with excessive interstitial pressure, lysis, necrosis, and proteases, which can be largely discounted using VEGF-transfected CHO cells. All of the modifications described in the tumor vasculature were not only reproduced in the VEGF:CHO tumor model, they were exaggerated; the percentage of fenestrated vessels was increased, and the CF-labeled fenestral diaphragms were decreased. To further simplify the system and to determine whether the generation of fenestrations in neovascular endothelium is a specific response to VEGF or generalized to all angiogenic growth factors, we used slow-releasing pellets incorporated with either VEGF or bFGF. Unexpectedly, neovascularization in response to bFGF had some fenestrated endothelium. However, the degree of fenestration is substantially higher with VEGF compared to bFGF, although the angiogenic response was greater with bFGF. Doses of VEGF and bFGF in the pellets were chosen to be sufficient for angiogenesis with negligible inflammatory responses (34, 71), but there were some infiltrating macrophages and plasma cells around the pellets. It is unclear whether bFGF induces fenestrations directly or indirectly by inducing: (a) VEGF expression (72); (b) infiltration of VEGF-producing cells (73, 74); or (c) similar transcription programs as VEGF.

Possibly the most intriguing result is that at doses too low to induce a visibly detectable angiogenic response, chronic secretion of VEGF was able to induce and maintain fenestrations in skeletal muscle microvascular endothelium. This finding further substantiates the role of VEGF in inducing and maintaining fenestrated endothelium in normal physiology (21, 75). Whether the natural endothelial fenestrae are the result of low concentrations of continuous VEGF165 secretion, other isoforms of VEGF, other factors entirely, supporting cells, such as pericytes, or a combination of these factors is unclear.

Data have been presented that peripheral tumor vascular endothelium has quantitatively more fenestrations and open junctions than normal vessels from which they are derived. The fenestral diaphragms are the result of low concentrations of continuous VEGF165 secretion, possibly the most intriguing result is that at doses too low to induce a visibly detectable angiogenic response, chronic secretion of VEGF was able to induce and maintain fenestrations in skeletal muscle microvascular endothelium. This finding further substantiates the role of VEGF in inducing and maintaining fenestrated endothelium in normal physiology (21, 75). Whether the natural endothelial fenestrae are the result of low concentrations of continuous VEGF165 secretion, other isoforms of VEGF, other factors entirely, supporting cells, such as pericytes, or a combination of these factors is unclear.

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REFERENCES


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W. Gregory Roberts and George E. Palade

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