Microenvironment and Immunology

Tumor-Surrogate Blood Vessel Subtypes Exhibit Differential Susceptibility to Anti-VEGF Therapy

Basel Sitohy, Janice A. Nagy, Shou-Ching Shih Jaminet, and Harold F. Dvorak

Abstract

Antivascular therapy directed against VEGF or its receptors (VEGFR) has been successful when administered at early stages of tumor vessel growth but is less effective when administered later. Tumor blood vessels are heterogeneous, so vessel subpopulations may differ in their requirements for tumor cell–secreted VEGF and in their susceptibility to anti-VEGF/VEGFR therapy. Human cancers contain several distinct blood vessel types, including mother vessels (MV), glomeruloid microvascular proliferations (GMP), vascular malformations (VM), feeding arteries (FA), and draining veins (DV), all of which can be generated in mice in the absence of tumor cells using expression vectors for VEGF-A164. In this study, we investigated the sensitivity of each of these vessel types to anti-VEGF therapy with Aflibercept (VEGF Trap), a potent inhibitor of VEGF-A164. Administering VEGF Trap treatment before or shortly after injection of a recombinant VEGF-A164–expressing adenovirus could prevent or regress tumor-free neovascularization, but it was progressively less effective if initiated at later times. Early-forming MVs and GMPs in which the lining endothelial cells expressed high levels of VEGFR-2 were highly susceptible to blockade by VEGF Trap. In contrast, late-forming VMs, FAs, and DVs that expressed low levels of VEGFR-2 were largely resistant. Together, our findings define the susceptibility of different blood vessel subtypes to anti-VEGF therapy, offering a possible explanation for the limited effectiveness of anti-VEGF/A/VEGFR treatment of human cancers, which are typically present for months to years before discovery and are largely populated by late-forming blood vessels.

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Introduction

Most tumors need to induce the formation of new blood vessels if they are to grow beyond minimal size (1). They are thought to do so, primarily, by expressing VEGF-A and particularly VEGF-A164 (mouse; human VEGF-A165; refs. 2, 3). VEGF-A initiates signaling by binding to specific, plasma membrane–associated receptors on vascular endothelial cells, particularly VEGF receptor-2 (VEGFR-2; Flk-1; ref. 4). Accordingly, VEGF and its receptors have been regarded as attractive therapeutic targets. Antibodies against VEGF-A, as well as tyrosine kinase inhibitors directed against VEGFR-2, are effective as monotherapy against many rapidly growing mouse tumors (3); however, they have been of less benefit when used in treating cancer patients. Bevacizumab (Avastin; Genentech, South San Francisco, CA), a humanized anti-VEGF-A antibody, was approved for use in combination with chemotherapy (5), and has not lengthened life expectancy in several other cancers (6).

It has been known for some time that tumor blood vessels are not of a single type (7), and this heterogeneity raises the possibility that different types of tumor blood vessels might be differentially sensitive to anti-VEGF or anti-VEGFR therapy. Human tumors are typically present for months or years before discovery and therefore could be supplied by a different population of blood vessels than rapidly growing transplantable mouse tumors. Thus, the difference in outcome between many human and mouse cancers could reflect differences in blood vessel type, their differential requirements for tumor-secreted VEGF-A, and therefore their differential sensitivity to anti-VEGF/VEGFR therapies.

Testing this hypothesis requires detailed knowledge of the different types of blood vessels present in tumors and their properties, and few attempts have been made to classify tumor blood vessels rigorously (7). Recently, we categorized the different blood vessels present in several important human cancers (8, 9) and identified the following structurally distinct abnormal vessel types: mother vessels (MV), glomeruloid microvascular proliferations (GMP), and vascular malformations (VM). Of these vessel types, GMPs were most prominent in glioblastoma multiforme, but the other vessel types were all prominently represented in human colon, kidney, and ovarian cancers (8, 9). In addition, highly abnormal, enlarged feeding arteries (FA) and draining veins (DV) supply and drain the angiogenic, intratumor and immediately paratumor blood vessels, that is, MVs, GMPs, and VMs.

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Investigating the differential susceptibility of these vessel subtypes to anti-VEGF/VEGFR therapies in human cancer patients is difficult. Therefore, we developed an animal model in which each of these blood vessel types is replicated in a highly reproducible manner in nude mice in the absence of tumor cells using an adenoviral vector that expresses VEGF-A164 (10). This reductionist mouse model has allowed us to analyze the sequential steps and mechanisms by which each type of new pathologic blood vessel forms and to characterize their functional properties (10). MVs, GMPs, and VMs developed from preexisting postcapillary venules by a process of angiogenesis, whereas FAs and DVs developed by arteriogenenesis from preexisting arteries and veins.

The present studies were undertaken to determine which of the different tumor-surrogate blood vessel types induced by Ad-VEGF-A164 were or were not susceptible to anti-VEGF-A therapy. To that end, we used Aflibercept (VEGF Trap), a human soluble decoy receptor protein created by fusion of portions of VEGFR-1 and VEGFR-2 to the Fc segment of IgG1 (11). VEGF Trap has high affinity for all of the isoforms of VEG-A, VEG-B, and placental growth factor, but it does not bind VEG-C. We now report that VEGF Trap potently inhibited both angiogenesis and arteriogenenesis when administered prior to Ad-VEGF-A164 and at least partially regressed angiogenic and arteriogenetic blood vessels when administered up to several weeks thereafter. However, VEGF Trap was without significant effect when therapy was delayed until 2 months after Ad-VEGF-A164 when “late” vessels, VMs, FAs, and DVs predominated. The endothelial cells lining these late vessels, in contrast to those of the highly susceptible MVs and GMPs, expressed little or no VEGFR-2. These findings could have important implications for understanding the limited effectiveness of anti-VEGF/VEGFR treatments in human cancer, where “late” blood vessels that have acquired independence from tumor-secreted VEGF-A are likely to predominate.

Materials and Methods

Animal model, treatment with VEGF Trap, and histology

A total of 10⁸ plaque-forming units (PFU) of Ad-VEGF-A164 in 10 μL PBS/3% glycerol were injected into the ears of female athymic nude mice (National Cancer Institute) and 5 × 10⁶ PFU in 50 μL PBS/3% glycerol were injected into flank skin (12). Animals were injected subcutaneously with Aflibercept (VEGF Trap, 25 mg/kg, Regeneron) or equivalent control peptide (human Fc fragment that serves as the backbone of VEGF Trap) as in Table 1 (11). Twelve or more mice, equally matched, were used per group. Ears and flanks were viewed and photographed in a dissecting microscope. All animal studies were approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center, Boston, MA.

Histology and immunohistochemistry

For histology, tissues were fixed in K2 fixative and embedded in Epon for Giemsa-stained, 1 μm-Epon sections (13). Immunohistochemistry was carried out on 4% paraformaldehyde-fixed, paraffin-embedded tissues with antibodies to VEGFR-2 as previously described (13).

### Table 1. VEGF Trap/Control treatment regimens

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment days (VEGF Trap or control peptide)</th>
<th>Harvest day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>−1, +2</td>
<td>5</td>
</tr>
<tr>
<td>Group 2</td>
<td>+5, 8, 11</td>
<td>12</td>
</tr>
<tr>
<td>Group 3</td>
<td>+11, 14, 17, 20</td>
<td>21</td>
</tr>
<tr>
<td>Group 4</td>
<td>+21, 24, 27, 30</td>
<td>31</td>
</tr>
<tr>
<td>Group 5</td>
<td>+51, 54, 57, 60</td>
<td>61</td>
</tr>
</tbody>
</table>

Vascular casting with Microfil

Mice anesthetized with avertin (200 mg/kg) and injected intravenously with 100 units of heparin were perfused through the left ventricle with 60 mL prewarmed, heparinized saline at 80 mm Hg, followed by sufficient Microfil (MV-122, Flow Tech, Inc.) at 120 mm Hg to fill the entire circulatory system. After 18 to 24 hours at 4°C, tissues were dehydrated in glycerol as per manufacturer’s instructions and photographed.

Quantification of intravascular plasma volume and plasma leak

We improved a 2-tracer method to quantify intravascular plasma volume and plasma albumin leakage, measures of total blood vessel formation and vascular permeability, respectively (12). At time zero, mice were injected intravenously with 0.1 mL of 0.5% Evans blue dye, which binds to plasma albumin. Immediately thereafter, a sample of blood (100 μL) was taken by retro-orbital puncture into 10 μL of heparin (1,000 units per mL). Twenty-five minutes later, a second intravenous injection was administered, this time of 0.1 mL of Hanks’ balanced salt solution containing 0.1% bovine serum albumin as carrier, and another sample of blood (100 μL) was taken immediately by retro-orbital puncture into 10 μL of heparin (1,000 units per mL). Five minutes later, at time 30 minutes, mice were euthanized and Ad-VEGF-A164 injection sites were carefully harvested to minimize blood loss (8-mm punch biopsy for ear samples and 10-mm punch biopsy for flank samples), weighed, and subjected to γ-counting. The collected blood samples were centrifuged (14,000 rpm, 10 minutes) to pellet blood cells, and the plasma was removed and stored at 4°C. Tissues were extracted in 1 mL of formamide for at least 48 hours at 56°C. The absorbance at 620 nm of the tissue extract was determined by spectrophotometry and converted to the amount (micrograms) of Evans blue tracer in each sample using a standard curve. The concentrations of Evans blue dye and 125I-albumin in the corresponding plasma samples were also determined by spectrophotometry and converted to the amount (micrograms) of Evans blue tracer in each sample using a standard curve. The concentrations of Evans blue dye and 125I-albumin will have had time to extravasate at 5 minutes after injection. Therefore, the 125I-albumin value at 5 minutes provides a quantitative measure of intravascular plasma volume, whereas the Evans blue albumin value at 30 minutes provides a...
measure of the sum of both intravascular and extravascular plasma albumin volume. The volume of plasma albumin that has extravasated can then be determined by subtracting the 5-minute value from the 30-minute value.

Statistics
Data are presented as mean ± SEM. Statistical analyses were conducted with InStat 3 software (GraphPad), using the non-parametric Mann–Whitney U test.

Results
Effect of VEGF Trap on the neovascular response induced by Ad-VEGF-A₁₆₄
Ad-VEGF-A₁₆₄ induces the formation of tumor-surrogate blood vessels in nude mice by 2 parallel processes: angiogenesis and arteriovenogenesis (9, 10). The response begins within a day, evolves over a period of weeks to months, and leaves behind a substantial number of newly formed blood vessels that persist indefinitely (>1 year). VEGF Trap (or control peptide) was administered at various times before and after Ad-VEGF-A₁₆₄ injection (Table 1). Ad-VEGF-A₁₆₄ injected into mice treated with control peptide induced a strong neovascular response in both ears and flank skin that was identical to that in mice receiving Ad-VEGF-A₁₆₄ alone (Fig. 1). VEGF Trap profoundly diminished the macroscopic vascular response induced by Ad-VEGF-A₁₆₄ in groups 1 to 4 mice but had no apparent effect on group 5 mice (Fig. 1). Mice in none of the groups exhibited any signs of toxicity.

Effect of VEGF Trap on the different subtypes of new blood vessels induced by Ad-VEGF-A₁₆₄
In both ears and flank skin, MVs are the first new angiogenic vessel subtype to form (9, 10). They are greatly enlarged, pericyte-poor, hyperpermeable blood vessels that develop from preexisting normal venules as the result of basement membrane degradation and pericyte detachment (9, 10, 14). MVs peak on day 5, at the time of maximal intravascular volume and plasma protein leakage [Figs. 2, control peptide (C) group 1, and 3A]. Consistent with their hyperpermeability to plasma proteins, injection sites exhibited extensive edema (Fig. 2, group 1 control). Subsequently, MVs evolved over 4 to 6 weeks into several distinct types of “daughter” blood vessels, including GMPs and VMs (Fig. 2, control group 4). GMPs are poorly organized vascular clusters that resemble renal glomeruli macroscopically (hence their name) and are composed of pericyte-coated, endothelial cell–lined, minimally sized vascular channels with reduplicated basement membranes (Fig. 3C; refs. 9, 10). VMs are large, tortuous vessels that derive from MVs by acquiring an often asymmetrical smooth muscle coat and are the only angiogenic vessel subtype that persists beyond 2 months (Fig. 3E; refs. 9, 10). Arteriovenogenesis develops in parallel with angiogenesis as preexisting small arteries and veins remodel and enlarge to form FAs and DVs that supply the angiogenic vasculature and, like VMs, persist indefinitely (Figs. 1, 2, and 3E and F). At 61 days and later times, VMs, FAs, and DVs were the predominant Ad-VEGF-A₁₆₄-induced blood vessel types present (Fig. 2, group 5).

VEGF Trap profoundly affected Ad-VEGF-A₁₆₄-induced angiogenesis and arteriovenogenesis, but its effectiveness was highly dependent on the time of administration. When injected prior to Ad-VEGF-A₁₆₄, MV formation, vascular permeability and edema were largely prevented [Figs. 2, treated (T) group 1, and 3B]. When injected up to one month after Ad-VEGF-A₁₆₄ injection, VEGF Trap–treated mice exhibited only rare, small GMPs, fewer VMs, and smaller FAs and DVs, and considerably less edema than controls (Figs. 2, T group 4, and Fig. 3D). However, when treatment was delayed for 2 months (group 5),
VEGF Trap had almost no inhibitory effect. VMs, FAs, and DVs were indistinguishable from controls in VEGF Trap–treated mice (Fig. 3E and F) and both treated and control groups developed an equivalent mast cell–rich, fibrous connective tissue stroma, similar to that found in desmoplastic tumors (Fig. 2, group 5).

**VEGFR-2 (Flk-1) expression levels in different blood vessel types induced by Ad-VEGF-A**

A possible reason for the failure of late vessels to respond to VEGF Trap is a loss of their dependence on exogenous (i.e., Ad-VEGF-A–induced) VEGF-A. To test this possibility, we carried out immunohistochemistry on the different vessel types to assess changes in VEGFR-2 expression. MV and GMP endothelial cells stained strongly for VEGFR-2 whereas those lining VMs, FAs, and DVs stained weakly or not at all (Fig. 4).

**Quantification of intravascular volume and vascular leak**

We refined a double tracer method that we had developed earlier (12) to provide an unbiased, quantitative measure of both the extent of new blood vessel formation (intravascular volume) and the extent of vascular leakage. This method is superior to measures evaluating vascular density for quantifying angiogenesis in that it takes into account the entire vasculature within a prescribed tissue volume, thus avoiding the problems of vascular variability in different regions and the associated risk of sampling bias. It is also superior to the classic Miles assay in that it clearly distinguishes between dye–albumin complex that is within versus that which is outside the vasculature. Using this method, we showed that Ad-VEGF-A induced a dramatic increase in intravascular plasma volume in both ear and flank skin of mice receiving control peptide (Fig. 5); peak levels were observed on day 5, at the height of MV formation, and persisted thereafter, though at somewhat lower levels. By day 61, intravascular plasma volume remained significantly elevated, approximately twice that of normal plasma volumes in both ear and flank skin. Leakage of plasma albumin was also dramatically increased in Ad-VEGF-A–injected mice receiving control peptide; again maximum leakage was observed at 5 days and declined progressively thereafter until little (ears) or no (flank skin) leakage was detected at 31 days and none at 61 days (both ears and flank skin). This was expected as MVs and GMPs, hyperpermeable blood vessels, had by that time been largely replaced by "late" vessels (VMs, FAs, and DVs) that are not hyperpermeable (9, 10).

VEGF Trap substantially reduced both intravascular plasma volume and plasma albumin leak in both ear and flank skin of groups 1 to 4 mice (Fig. 5). However, at 61 days, intravascular plasma volumes in both ears and flank skin of group 5 control peptide mice remained elevated approximately 2-fold above those of normal levels, and these volumes were not significantly diminished by VEGF Trap.

**Discussion**

VEGF Trap effectively prevented both angiogenesis and arteriovenogenesis when administered prior to injection of Ad-VEGF-A. In group 1 VEGF Trap–treated mice, new blood vessels largely failed to develop and increases in intravascular plasma volume, permeability, and edema were minimal (Figs. 1–3 and 5). When VEGF Trap was instead administered at intervals up to one month after Ad-VEGF-A injection, the MVs, GMPs, and early VMs that had formed regressed, and
vascular volumes and associated leakage were greatly reduced; arteriovenogenesis was also inhibited. However, VEGF Trap was largely ineffective when treatment was delayed until 2 months after Ad-VEGF-A164 injection (Figs. 1–3 and 5). At this late time, angiogenesis had largely passed through the stages of MVs and GMPs to form fully developed VMs, and arteriovenogenesis had proceeded to form well-developed FAs and DVs. Thus, VEGF Trap prevented new blood vessel formation and efficiently regressed "early" blood vessels (MVs and GMPs) induced by Ad-VEGF-A164; however, it had essentially no effect on the "late" blood vessels induced either by angiogenesis (VMs) or arteriovenogenesis (FAs and DVs). VEGF-A delivered to endothelial cells by a paracellular mechanism from closely apposed smooth muscle cells would likely not be accessible to VEGF Trap. Evidence in favor of this possibility was obtained from immunohistochemistry; MVs and GMPs expressed extremely high levels of VEGFR-2 staining, whereas VMs, FAs, and DVs expressed very low or undetectable levels (Fig. 4).

Figure 3. Higher magnification of the different types of blood vessels induced by Ad-VEGF-A164, following treatment with VEGF Trap or control peptide. MVs (A and B) and GMPs (C and D) are much larger and more frequent in control peptide–treated than in VEGF Trap–treated mice, whereas once-formed, VMs, FAs, and DVs are not distinguishable (E and F). h, hair follicle; L, lymphatic; N, nerve. Magnification bars, 25 μm.

One possible explanation for the insensitivity of VMs, FAs, and DVs to VEGF Trap is that their lining endothelial cells have lost dependence on exogenous VEGF-A and that any requirement for VEGF-A as a survival factor has been met by the pericytes or smooth muscle cells that closely envelop them. Insufficient attention has been paid in the literature to the heterogeneity of the tumor vasculature and to the origins and specific properties of the different types of tumor blood vessels and their susceptibility to anti-VEGF/VEGFR therapy. A number of reports indicate that vessel types variously described as "pericyte-coated," "α-smooth muscle actin (α-SMA)–positive," "mature," "normalized," "stable," "established," or "large" are resistant to such therapy in mouse and/or human cancers (16–22), whereas vessels described as "immature," "dilated," or "unstable" are sensitive (17, 23–25). However, there are exceptions to this pattern of differential sensitivity, as VEGF Trap has been reported to regress smooth muscle–coated blood vessels in at least some tumor models (26, 27). In any event, the specific types of resistant or sensitive vessels, their mode of formation, their detailed properties, and their relationships to the vascular categories we have described in human tumors and in the tumor-surrogate vessels induced by Ad-VEGF-A164 have not previously been well-defined.
Our results with VEGF Trap agree with reports of anti-VEGF/VEGFR therapy in a number of different mouse cancers. Anti-VEGF antibodies and/or small molecule VEGFR inhibitors have been found to inhibit the growth of many transplantable, VEGF-secreting mouse tumors and tumor xenografts (3, 17, 28, 29). Review of the literature and our own data indicate that MVs are the predominant vessel type, at least during early stages of growth, in at least the following tumors, many of which have been treated effectively with anti-VEGF/VEGFR approaches: mouse B16 melanomas, Lewis lung carcinomas, MOT, TA3/St mammary carcinoma, guinea pig line 1 and 10 bile duct carcinomas (refs. 30, 31; our unpublished data), and, as best as can be determined from published photomicrographs, early stages of the autochthonous Rip-Tag2 tumor (29). Therefore, it may be safely presumed that MVs are the primary vessel subtype susceptible to anti-VEGF/VEGFR treatment. Additional strong evidence for this conclusion comes from the finding that anti-VEGF/VEGFR drugs potently inhibit tumor vascular hyperpermeability (24, 32, 33). In the pathologic angiogenesis induced by tumors or Ad-VEGF-A164, MVs (and to a lesser extent GMPs) are the only hyperpermeable blood vessels (9, 10).

In contrast to their effectiveness in early mouse tumors populated largely by MVs, anti-VEGF/VEGFR therapies have been much less effective at later stages of growth in mouse tumors and have had only limited effectiveness in treating human cancers (5, 6). One explanation is that tumor cells exposed to these therapies begin to express new growth factors such as members of the fibroblast growth factor family, angiopoietins, and interleukin-6, thereby circumventing the need for VEGF-A (17, 28, 34, 35). However, an additional explanation is that, over time, the tumor vasculature evolves to form "late" blood vessels (VMs, FAs, and DVs) that are not susceptible to VEGF/VEGFR inhibition. Human cancers in particular are commonly present for many months or years before they are discovered and so have sufficient time to form large numbers of "late" blood vessels. Quantification of the relative numbers of each vessel type in resected human carcinomas is an impossible task, but we have shown that, at the very least, important human carcinomas (e.g., colon, breast, ovary, and kidney) contain large numbers of VMs, FAs, and DVs (8, 9).

Our data also offer an alternative interpretation of the "normalizing" effect that anti-VEGF/VEGFR therapies have on the tumor vasculature. Normalization refers to the decrease in vascular permeability, edema, and interstitial tissue pressure induced by anti-VEGF/VEGFR therapies (36, 37). We suggest...
that these therapies, as here with Ad-VEGF-A\textsuperscript{164}–induced angiogenesis, selectively attack MVs (and GMPs when present), as these are the only tumor blood vessels known to be hyperpermeable to plasma proteins; edema and increased interstitial hyperpermeability, of course, result from increased vascular permeability. Left behind, then are relatively unaffected pericyte- and smooth muscle–coated VMs, FAs, and DVs; that is, vessels that by radiological examination and immunocytochemistry seem relatively normal in that they are smooth muscle cell coated, smaller on average than MVs, and not hyperpermeable to circulating macromolecules.

In summary, we have shown that specific, well-defined subsets of VEGF-A–induced blood vessels (MVMS and GMPs) are sensitive to VEGF Trap therapy whereas other vessel subsets (VMs, FAs, and DVs) are not. Because VEGF Trap potently binds to and inactivates VEGF-A, these data are likely representative of data obtained with Avastin or VEGFR inhibitors and thus may help to explain the limited benefit of anti-VEGF/VEGFR therapy in treating human cancers. We suggest that antivascular therapy could be improved by finding new targets on late tumor blood vessels, that is, those vessels that are not susceptible to anti-VEGF/VEGFR therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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