Localization of vascular response to VEGF is not dependent on heparin binding

Matthew L. Springer,*1 Andrea Banfi,†2 Jianqin Ye,* Georges von Degenfeld,†3 Peggy E. Kraft,† Shereen A. Saini,* Neel K. Kapasi,* and Helen M. Blau†

*Department of Medicine, Division of Cardiology, University of California, San Francisco, San Francisco, California, USA; and †Baxter Laboratory in Genetic Pharmacology, Departments of Molecular Pharmacology and of Microbiology and Immunology, Stanford University, Stanford, California, USA

ABSTRACT The major vascular endothelial growth factor (VEGF) isoforms are splice variants from a single gene that differ in their extent of heparin affinity due to the absence of the heparin binding domain in the smallest isoform (mouse VEGF120, human VEGF121). A long-held assumption that has guided the use of VEGF isoforms clinically has been that their differences in heparin binding dictate their ability to diffuse through tissue, with VEGF121 moving most freely and that the distribution of recombinant VEGF would have therapeutically relevant consequences. To test this assumption, we delivered the genes encoding these isoforms by myoblast-mediated gene transfer, a means of delivering genes to highly localized sites within muscle. Surprisingly, all isoforms induced comparable extremely localized physiological effects. Significantly, irrespective of the isoform delivered, the vessels passing within several micrometers of muscle fibers expressing VEGF displayed sharply delineated changes in morphology. The induction of capillary wrapping around VEGF-producing fibers, and of vascular malformations in the muscle at high levels, did not differ among isoforms. These results indicate that heparin binding is not essential for the localization of VEGF in adult tissue and suggest that the preferential delivery of VEGF121 cDNA for clinical applications may not have a physiological basis.—Springer, M. L., Banfi, A., Ye, J., von Degenfeld, G., Kraft, P. E., Saini, S. A., Kapasi, N. K., Blau, H. M. Localization of vascular response to VEGF is not dependent on heparin binding. FASEB J. 21, 2074–2085 (2007)

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Vascular endothelial growth factor (VEGF-A, referred to here as VEGF), one of the major regulators of angiogenesis, has long been known to exist in multiple isoforms due to alternate mRNA splicing (1–3). The three major isoforms each differ in length between rodents and humans by one amino acid, with VEGF188/189 (rodent/human) encoded by exons 1–8, VEGF164/165 missing exon 6, and VEGF120/121 missing exons 6 and 7 (for a review, see reference 4). Although a number of early studies demonstrated that this differential splicing corresponded to differential heparin affinity due to the partial and complete absence of a heparin-binding domain in 164/165 and 120/121, respectively (5–7), an understanding of the biological role of this diversity is still far from complete.

In recent years, significant advances have been made toward the elucidation of the respective roles of the VEGF isoforms in development using transgenic mouse models that express only one or several specific isoforms (8–16). In addition, a number of studies have been carried out that examine the relationship of specific isoforms to tumorigenicity (e.g., ref. 17). Despite the observation that all three of the main isoforms are angiogenic when the genes encoding them are exogenously expressed in ischemic limbs (18), little is known about how the specific isoforms influence the nature of the blood vessels that they induce in such a potentially therapeutic adult setting. This is important since clinical applications have been largely based on the assumption that the different isoforms bind extracellular matrix to different extents, which impacts their diffusion through tissue and their localization, and leads to distinct physiological effects. Although there is evidence in support of this concept during embryonic development, e.g., apparent isoform-specific differences in vessel branching patterns (12, 15), it has not yet been demonstrated in an adult setting. Nonetheless, human VEGF121 has been specifically chosen over the more prevalent VEGF165 for some clinical trials of VEGF gene therapy (19–21), based in part, on its expected diffuse distribution in tissue that would avoid steep concentration gradients and provide larger regions of angiogenic effects.

In this study, we have used a highly localized cell-
mediated gene delivery approach to determine whether the nature and localization of vascular growth differ in response to expression of mouse VEGF120, 164, and 188. In particular, because the role and relevance of the different VEGF isoforms appear to be tissue specific (17), we sought to determine the effects of their delivery to skeletal muscle because this is one of the main target tissues for gene therapy approaches for the treatment of ischemic conditions (22). We have previously described a VEGF gene delivery system in which primary mouse myoblasts engineered to express VEGF are implanted in mouse skeletal muscle, where they fuse to preexisting muscle fibers and cause them to constitutively overproduce VEGF (23, 24). Because the myoblasts do not travel far from the site of implantation in muscle, the resulting VEGF-expressing region of muscle is localized to a much greater degree than in similar approaches using direct viral transduction of the muscle (25, 26). This cell-mediated delivery approach has enabled us to study the interface of VEGF-expressing and nonexpressing regions in mouse hind limb skeletal muscle and to demonstrate that localized clusters of capillaries at the site of VEGF production are surrounded by an adjacent zone of arteriole formation (27). In related studies, we have reported that VEGF 164 exerts its influence on capillaries at a microenvironmental level, causing highly localized changes in vessel morphology over just a few micrometers (28) that affects the ability of the vessels to increase blood flow to ischemic tissue (29). We hypothesized that myoblast-mediated delivery of the nonheparin binding VEGF120 would result in significantly reduced localization of effects on vessel morphology. Surprisingly, we report here that when the genes encoding the three main VEGF isoforms are directly compared by expression in the same adult mouse muscle and by the same gene delivery approach, the effects on vessel morphology are similarly highly localized regardless of heparin affinity. Furthermore, the growth patterns and morphology of the influenced vessels are similar in all three cases, including vessel wrapping around VEGF-producing muscle fibers, increased vessel size and tortuosity, formation of glomeruloid bodies, and hemangioma formation on high-level, long-term production. These data indicate that heparin binding efficiency alone does not dictate the localization of VEGF-induced effects and that other extracellular matrix and receptor interactions are likely to play a more important role in VEGF bioavailability. Furthermore, clinical delivery of human VEGF121 will likely result in a similar protein distribution as VEGF165, and will not obviate problems stemming from VEGF localization and microenvironmental influences.

MATERIALS AND METHODS

Construction of VEGF isoform retroviruses and VEGF-expressing myoblasts

A plasmid that contains the MFG retroviral vector with a mouse VEGF164 cDNA transgene (23) was digested with BclI and BamHI, excising a 473 bp fragment, including the 3′ region of the VEGF164 coding sequence. The remainder of the plasmid was ligated to comparable BclI-BamHI fragments isolated from plasmids encoding mouse VEGF120 (337 bp fragment) and VEGF188 (539 bp fragment) (plasmids were generous gifts from P. D’Amore). These fragments included the entire region of difference between the three isoforms, thus generating MFG retroviral plasmids encoding all three VEGF isoforms. DNA sequencing was performed to confirm the expected sequences of the three different variants. Infectious, nonreplicating retrovirus was generated from these plasmids, and mouse primary myoblasts were transduced as described previously (30). Myoblasts were cultured at 5% CO2 on collagen-coated dishes, as described previously (31, 32).

Immunostains of myoblasts in culture

For immunostaining in culture, myoblasts were incubated for 2.5–3 h in growth medium containing 100 μm/L monensin to block secretion (30) and were then immunostained as described previously (35). The primary antibody was a rabbit polyclonal antibody against VEGF (ab173, Abcam, Cambridge, UK; note: this antibody is no longer available) used at 1:100, and the secondary antibody was Texas Red-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR, USA) used at 1:100. Hoechst 33258 nuclear dye was included with the secondary antibody at 0.12 μg/ml. Negative controls without primary antibody were always included. Cells were rinsed with PBS and examined by fluorescence microscopy in the dish using a water immersion ×40 objective.

For ELISA measurements, cells were incubated in fresh culture medium with 100 μm/L monensin for 3 h, scraped into a microcentrifuge tube, and centrifuged. The pellet was frozen in liquid N2 stored, and subsequently thawed into lysis buffer containing 1% Triton X-100, 20 mmol/L Tris pH 8.0, 117 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, and a cocktail of protease inhibitors. The extract was centrifuged in a microcentrifuge, and the supernatant was assayed for protein content by the Bradford assay and adjusted to 5 mg/ml. The extract was analyzed in duplicate at several dilutions using a mouse VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA); no pair of duplicate measurements differed by more than 7%. Because VEGF164 protein was used as the ELISA standard and VEGF120 and 188 are smaller and larger peptides than 164, respectively, ELISA results based on monoclonal antibody binding will overestimate the number of micrograms of VEGF120 and underestimate the number of micrograms of VEGF188. However, when comparing the delivery of the three isoforms, we reasoned that molar quantity was the important factor as opposed to total weight. Therefore, final ELISA results were expressed as VEGF164-equivalent ng/ml, a convenient representation of moles/ml, and used to calculate numbers of implanted myoblasts. Results were also expressed as pg(164-equivalent)/mg total cellular protein.

Myoblast implantation

Mice were obtained from Taconic (Germantown, NY, USA) and treated in accordance with the guidelines of the Stanford University Administrative Panel on Laboratory Animal Care. Cell extracts taken from myoblasts cultured for a constant length of time were assayed for VEGF production by ELISA. Readings were normalized to account for the different molecular weights of the different isoforms, as described above, and cell numbers for implantation were calculated to result in implantations with equivalent total VEGF production. Six- to eight-week-old male SCID C.B-17 mice were anesthetized with...
methoxylurane (Medical Developments, Springvale, Australia). After trypsinization and preparation for implantation, as described by Rando and Blau (32), 5 μl of cells in PBS with 0.5% BSA were implanted once into each tibialis anterior muscle and once into each lateral gastrocnemius muscle in the following numbers per injection using a 30-gauge needle: 1 × 10^6 of control cells expressing only lacZ, 4 × 10^6 of VEGF120-expressing cells, 1 × 10^5 VEGF164 cells, and 1.5 × 10^5 VEGF188 cells. Nine legs were implanted per cell group. At three time points consisting of 7 days, 14 days, and 48 days postimplantation, 6 mice spanning all four leg groups were euthanized and their legs (postimplantation 6 mice spanning all four leg groups were harvested and frozen in isopentane/liquid nitrogen for cryosectioning.

Other legs were implanted with cells expressing the different isoforms in similar fashion for RT-PCR confirmation of VEGF isoform expression. Cells were injected into the tibialis anterior at two different sites per leg, two legs per mouse, two mice per isoform. One week later, the tibialis anterior muscles were harvested, the two legs from each mouse were combined, and the muscle was processed for RNA extraction as described below.

For myoblast implantation into ear muscle, 10 mice were anesthetized with Avertin and injected into the posterior auricular muscle mid-way up the dorsal aspect of the external ear using a 29.5-gauge needle as described by Ozawa et al. (28). Of the resulting 20 ears that were injected, myoblasts were implanted in 5 ears per group. Cell numbers for implantation were identical to those used for hindlimb muscle implantations, described above, unless otherwise specified. After 14 days, mice were intravenously injected with lectin and processed as described below.

RT-PCR

Muscle was homogenized using a Polytron homogenizer in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) on ice, according to the manufacturer’s instructions. Reverse transcription was carried out using a Superscript III First-Strand Synthesis kit (Invitrogen) from 1.5-μg template cDNA using an Advantage 2 PCR kit (Clontech, Mountain View, CA, USA) with the following strategy: 95°C 3 min, (95°C denaturation 30 s, 68°C annealing/extension 3 min) × 30–35 cycles, 68°C 10 min. Viral vector primers were 5′-ATTACACGCGGCCAGCTGTG-3′ forward and 5′-GCCCTG-GACCAGTGATATG-3′ reverse. Endogenous VEGF primers were 5′-CCTCGGATTCGGACCCGGG-3′ forward and 5′-GACCCAAATGTGCTCATGATG-3′ reverse. The three VEGF-encoding isoforms in similar fashion for RT-PCR confirmation of VEGF isoform expression. Cells were injected into the tibialis anterior at two different sites per leg, two legs per mouse, two mice per isoform. One week later, the tibialis anterior muscles were harvested, the two legs from each mouse were combined, and the muscle was processed for RNA extraction as described below.

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Tissue processing and analysis

Tissue sections were cut at 20 μm for X-gal staining and at 10 μm for hematoxylin/eosin (H&E) staining and immunostaining. Sections were prepared for immunostaining as described by Springer et al. (27), using a rat monoclonal antibody against murine PECAM-1 (clone MEC 13.3; PharMingen, San Diego, CA; 1:100 dilution), a mouse monoclonal antibody against smooth muscle actin (clone 1A4; ICN Biomedicals, Aurora, OH, USA; 1:400 dilution), a rabbit polyclonal antibody against β-galactosidase (Eppendorf; –5 Prime, Inc., Boulder, CO, USA; note: this antibody is no longer being supplied), or a rabbit polyclonal antibody against VEGF (ab173, Abcam, Cambridge, MA, USA; 1:100). To quantitate localization of effect on vasculature, representative immunostained tissue sections were photographed using Openlab software (Improvision, Lexington, MA, USA) and coded for blinded analysis, and Imagej software (NIH) was used to measure the area in each picture occupied by β-galactosidase immunostaining and the area bounded by altered vasculature as judged by PECAM-1 immunostaining. The PECAM-1 and β-galactosidase regions were measured independently from each other. The area of altered vasculature was divided by the area of β-galactosidase staining and multiplied by 100 to result in a percent altered vasculature per transgenic region.

For processing and staining of ears, mice were injected intravenously with biotinylated Lycopersicon esculentum lectin followed by intravascular fixation and ear whole-mount staining, as described previously (28). Three-dimensional vascular morphology was revealed by HRP/DAB staining of the biotinylated lectin and transgenic muscle fibers by X-gal staining.

For serum VEGF measurements, mice were anesthetized with methoxylurane, and blood samples were collected retro-orbitally and allowed to clot. Serum was isolated and VEGF ELISA measurements were carried out as described above for cell extracts.

Statistical analysis by ANOVA was carried out using SPSS software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Myoblasts transduced with VEGF isoform retroviruses express VEGF in culture

Mouse primary myoblasts already expressing the β-galactosidase reporter gene lacZ (32) were transduced with retroviruses to express murine VEGF120 or 188. The resulting cells, along with myoblasts from the same isolate that had previously been engineered in a similar fashion to express VEGF164 (23), were assayed by immunofluorescence to confirm production of VEGF in culture. Immunofluorescent staining of all three batches of VEGF/lacZ-expressing myoblasts (referred to as VZ-120 cells, VZ-164 cells, and VZ-188 cells) and negative control myoblasts that only expressed lacZ (Z cells) was performed using a polyclonal antibody that recognizes all three VEGF isoforms. The three VEGF-expressing batches stained positive for VEGF at near 100% of the populations, while the Z cells were uniformly negative (Fig. 1A).

To deliver similar total doses of VEGF, we measured the amount present in cell lysates from the three VEGF isoform-expressing myoblast populations and normalized the cell numbers for implantation. Lysates were used rather than culture supernatants to avoid complications from VEGF188-specific binding to cell surfaces (35). Respective cell extracts contained VEGF120 at 370 pg/mg total protein, VEGF164 at 1400 pg/mg, and VEGF188 at 965 pg/mg (expressed in VEGF164 equivalents; see Materials and Methods for definition and derivation).
Muscles implanted with myoblasts expressing different VEGF isoforms produce mRNA transcripts of the expected sizes

To confirm that we could deliver the specific VEGF isoform genes to skeletal muscle, the three populations of VEGF-producing myoblasts were implanted into hindlimb muscles, where they fused with preexisting muscle fibers, and mRNA from these muscles was analyzed by RT-PCR with PCR primers specific to viral vector sequences flanking the coding region. The primers detected bands at the expected sizes for the coding regions plus flanking sequences and did not detect a band in DNA isolated from unimplanted muscle (Fig. 1B), confirming that the implanted muscles expressed the correct isoforms. A very faint band was sometimes observed in the absence of reverse transcriptase, which was due to amplification of genomic viral sequences from traces of contaminating cellular DNA (not shown). However, the use of the same amplification conditions and number of cycles ensured that the robust signal detected in the complete reactions was safely attributable to transcribed mRNA, demonstrating that the genes were actively expressed in vivo, in agreement with the detection of VEGF protein by immunostaining in culture (Fig. 1A).

All three major VEGF isoforms induce similarly localized angiogenic effects

To determine whether the vascular response to VEGF120 was similar to the response to the other isoforms, the three kinds of VEGF-producing myoblasts, as well as control Z cells, were implanted into hindlimb muscles (n=9 per cell type). Three mice per group were euthanized after 7 days and another three after 14 days, and the resulting 6 hindlimbs per time point of each cell type were sectioned and stained to detect β-gal, capillaries, and arterioles (representative examples are shown in Fig. 2). The samples from the 7-day harvests were difficult to interpret because inflammation caused by the needle wound had not yet abated, even in the negative control Z myoblast implantations, as described previously (23). However, examination of the samples from the 14-day harvests revealed that the VEGF-expressing myoblasts induced the formation of clusters of capillaries and arterioles, whereas the implantation of negative control Z myoblasts had no observable effects on vessels or muscle morphology, in agreement with our previous results (23, 27). The inflammation that persisted at this point was a macrophage response to the VEGF that we have extensively described elsewhere (23, 36). Surprisingly, the effect of the three different VEGF isoforms on the tissue was very similar: highly localized extra capillaries intermingled with the transgenic muscle fibers at the implantation site and several extra arterioles nearby, regardless of heparin binding ability. This was confirmed by quantitation of the extent of affected vasculature per area of transgenic muscle fibers, which failed to detect a significantly larger area of angiogenic effect following implantation of the VEGF120 myoblasts compared with the longer, heparin-binding isoforms (Table 1). This was further reflected in a VEGF immunostaining pattern for VEGF120 that closely matched the β-gal immunostaining pattern for transgenic muscle fibers (Fig. 3), suggesting that the VEGF120 did not travel away from the fibers that produced it to any great extent.

Three more mice from each group in the hindlimb implantation experiment were euthanized 48 days after myoblast implantation, at a time when constitutive VEGF164 overexpression has been shown to lead to hemangioma formation (23, 27). The tissue was sectioned, stained, and examined for evidence of...
Implantation of Z myoblasts did not induce any perturbation in the muscle, even at this late time point, whereas all three VEGF isoforms were able to induce hemangioma formation similar to those already described to result from implantation of VEGF164-expressing myoblasts (23, 27) (Fig. 4). The sizes of the hemangiomas were quite variable (see Discussion), but the tissue organization was not detectably different in response to the different isoforms.

**Figure 2.** Similarity in vascular response to different VEGF isoforms in hindlimb muscle. Implantation of cells expressing different isoforms was assessed histologically in sets of neighboring tissue sections by X-gal staining for β-gal activity (A, D, H, and L), H&E staining (B, E, I, and M), and immunofluorescent staining (C, F, G, J, K, and N) for PECAM-1 in red, smooth muscle actin in green, and β-gal in blue. A–C) Control Z cell implantation resulted in no changes in tissue morphology other than occasional centrally located nuclei in regenerating fibers. Immunofluorescence revealed no extra capillaries or arterioles as assessed by PECAM-1 staining or smooth muscle actin staining, respectively. D–F) A similar set of three neighboring sections from a VZ-120 cell implantation, along with G) an additional example from another mouse in that group, showed a localized increase in capillaries surrounding the transgenic muscle fibers and a cluster of arterioles within a several-fiber radius that is more visible in G. H–K) Similar examples from a VZ-164 implantation; the example in H–J is progressing into the hemangioma stage and the example in K is not as advanced. L–N) A similar example from a VZ-188 implantation. Localization of VEGF’s effects are similar in the three groups. Scale bar = 100 μm for all panels.

**Three-dimensional blood vessel morphology exhibits sharply delineated changes on a cellular level in response to localized VEGF120 production**

To study the effects of the different VEGF isoforms on vessel morphology, myoblasts were implanted into skeletal muscle bundles in the ear (posterior auricular muscle). The mouse ear has the advantage that it is transparent and very thin, allowing three-dimensional analyses of vessel morphology in whole mounts (37).
The same cell densities were injected in the ear for each sample as used in the hindlimb implantations. Fourteen days later, mice were injected intravenously with biotinylated *Lycopersicon esculentum* lectin, which binds to the luminal surface of blood vessels (37), and ears were harvested and processed to visualize the lectin and stained for β9252-gal activity. Microscopic examination of the tissue yielded a three-dimensional view of the resulting vasculature in the ear, in which any changes in vasculature could be correlated with the muscle fibers that were expressing VEGF and lacZ.

Again, the unexpected result was that implantation of VZ-120 cells, expressing the smaller VEGF120 isoform that was expected to be less localized in the tissue, yielded results comparable to those induced by the larger isoforms (Fig. 5). Implantation of myoblasts expressing any of the three VEGF isoforms resulted in extremely localized changes in vessel morphology, with the appearance of tortuous capillaries that grew in close association with VEGF-producing muscle fibers and that changed sharply from normal to aberrant morphology when entering the region of VEGF production, in agreement with our previous results (28). Glomeruloid bodies where observed (38), as were early hemangiomas (see also Fig. 6). There was a trend toward increased branching of capillaries in ears implanted with VZ-120 cells, although the significance of this observation was difficult to determine (see Discussion). Significantly, vessel morphology again changed suddenly in the vicinity of VEGF120 production, as it did for the heparin-binding isoforms, demonstrating a surprisingly similar level of localization for the effects of the nonheparin-binding isoform.

### VEGF does not accumulate in the serum regardless of heparin affinity

We tested the possibility that differences in heparin affinity would alter the prevalence of the different isoforms in the serum. We have previously reported that implantation of VEGF164-expressing myoblasts into skeletal muscle does not lead to increased serum VEGF levels unless resulting hemangiomas become so large that there is significant tissue damage (23). Reasoning that this may also be due to the sequestration of VEGF, we collected pre-euthanasia blood samples at days 7 and 14 from the mice described above, whose leg muscles had been implanted with Z cells, VZ-120 cells, and VZ-164 cells. Serum was isolated, and VEGF levels were assayed by ELISA. There was no significant difference observed between the VEGF levels detected in the serum from the VEGF120, VEGF164, and negative controls (Fig. 7). Therefore, even the nonheparin-binding VEGF isoform did not accumulate in the blood at appreciable levels.

### Expression of recombinant VEGF isoforms does not appreciably induce up-regulation of endogenous VEGF

To determine whether the highly localized effect of recombinant VEGF120 expression was truly a response to VEGF120 or due to the up-regulation of endogenous heparin-binding isoforms, we performed RT-PCR analysis of the implanted muscle and looked for any perturbations in the endogenous VEGF expression patterns. We compared expression of endogenous

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**Table 1. Area of altered vasculature as a percentage of transgenic area**

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<thead>
<tr>
<th>Isoform</th>
<th>% Region Affected Vasculature</th>
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<tr>
<td>VEGF120 (n = 4)</td>
<td>120 ± 13.6</td>
</tr>
<tr>
<td>VEGF164 (n = 7)</td>
<td>136 ± 34.7</td>
</tr>
<tr>
<td>VEGF188 (n = 4)</td>
<td>111 ± 25.0</td>
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*P = NS*
isoforms from muscle implanted with each of the three isoforms individually, as well as negative controls consisting of muscle implanted with lacZ-expressing myoblasts, and unimplanted muscle. Four implantation sites per condition were combined for each sample. While real-time PCR would be required for exact quantitation of transcript levels, we were unable to design real-time PCR conditions that reliably distinguished between the three isoforms. We therefore performed standard PCR using primers that recognize only endogenous VEGF transcripts on equal amounts of template cDNA with appropriate loading controls and compared the results from both 28 and 30 PCR cycles to avoid misinterpretations due to saturation of signal on the gel (Fig. 8). We did not observe any detectable changes in the intensity of the endogenous VEGF164 and VEGF188 bands under any implantation conditions. Although we cannot rule out a minor undetectable up-regulation of heparin-binding VEGF, clearly the primary and marked physiological effects observed resulted from exogenous VEGF120 delivery. We therefore conclude that the localized effects on the vasculature after VEGF120 gene delivery was caused by the VEGF120.
DISCUSSION

We have reported here that despite differences in heparin binding affinity, delivery of genes encoding the three main VEGF isoforms to skeletal muscle resulted in similar effects on the vasculature, with regard to both localization and morphology. Contrary to expectation, VEGF120 and VEGF188 induced similar effects to those previously described for VEGF164. These included causing capillaries to wrap around VEGF-expressing muscle fibers, to become larger and more tortuous and to grow into glomeruloid bodies, as well as the ability to induce the formation of hemangiomas on constitutive high-level expression (23, 27, 28, 39). Especially striking was the sharp delineation between affected areas around VEGF-producing fibers and the nonaffected areas only scant micrometers away from those fibers, even in the case of the nonheparin-binding isoform.

These results are surprising, in that there are several reports to the contrary, that is, that VEGF isoforms are differentially localized. However, these apparent discrepancies can be explained by several factors that we discuss below; namely, the range of tissue-specific roles that the VEGF isoforms play in healthy adult tissue, the unique nature of VEGF’s role in embryonic development, and the distinction between normal and pathological processes.

The angiogenic region was similarly localized to the vicinity of the transgenic muscle fibers in the case of all three isoforms, indicating that the VEGF120 isoform did not induce more widespread vessel growth. Absence of heparin binding ability did not cause VEGF120 that was overproduced in the muscle to accumulate in the serum, despite our previous theory that heparin binding prevented overexpressed VEGF164 from reaching the bloodstream (36). From these data, it is apparent that differential heparin binding alone is not sufficient to localize VEGF in the tissue, at least not in the system described here, and that clinical delivery of human VEGF121 is unlikely to obviate issues of localization or microenvironmental effects seen with VEGF165.

These results are surprising, in that there are several reports to the contrary, that is, that VEGF isoforms are differentially localized. However, these apparent discrepancies can be explained by several factors that we discuss below; namely, the range of tissue-specific roles that the VEGF isoforms play in healthy adult tissue, the unique nature of VEGF’s role in embryonic development, and the distinction between normal and pathological processes.

First, the relative levels of the main VEGF isoforms vary naturally from tissue to tissue under normal physiological conditions, as well as after exogenous activation of endogenous VEGF gene expression, and as a result of constitutive expression of a genomic/cDNA hybrid that allows normal VEGF mRNA splicing (40–42). Thus, findings regarding VEGF isoform roles in
one tissue cannot necessarily be extrapolated to other tissues and are likely to be very different. VEGF protein localization does indeed appear to be influenced by heparin binding in cultured cells, with the 189 and 206 isoforms localized to the extracellular matrix (43). However, the biological environment of a culture dish lacks the complexity of a tissue. In contrast, under the more complex tissue conditions of nonangiogenic transformed pancreatic islets, it has been suggested that VEGF120 can be sequestered through mechanisms other than heparin binding. Specifically, VEGF120 and VEGF164 are expressed and sequestered in this tissue, and both are released by MMP9 as part of the angiogenic switch during tumorigenesis (44, 45). This finding suggests that nonheparin components of the extracellular matrix are involved in VEGF protein dynamics in at least some tissues.

Furthermore, attempts to determine the roles of the different VEGF isoforms have involved mostly the investigation of embryonic development and pathological processes. An isoform-specific VEGF gradient appears to guide growth of vascular networks during embryonic development, as described below (12, 15). However, development is a highly dynamic process, with physical and signaling mechanisms that are quite different from those that characterize maintenance of adult tissue. Therefore, studies of isoform differences in embryogenesis cannot be directly compared to those in adulthood.

This point is illustrated by impressive studies aimed at dissecting the roles of the VEGF isoforms in blood vessel development, made possible by the creation of transgenic mouse strains expressing only single VEGF isoforms. Interestingly, the vascular defects associated with the expression of only VEGF120 appear to be specific to certain organs and systems, notably in the heart, where they were characterized by capillaries that were irregular, tortuous, and slightly dilated (9), but also in the lung (8), bone (10, 14), kidney (11), brain (12), and retina (13). Mice expressing only VEGF188 were defective in retinal arteriole formation but venule development was unaffected (13). These studies demonstrate convincingly that the VEGF isoforms are not interchangeable during embryonic development and that the balance of these splice variants is likely to be as important as the balance between VEGF-A and other growth factors in the establishment of a stable vascular system (46). In fact, it has been proposed that stable angiogenesis would benefit from interventions that allow the balance of VEGF splice variants to be determined by endogenous regulation (41, 47, 48), serving as a reminder of the diversity of potential tissue-specific responses to angiogenic stimuli.

These models have been used to demonstrate that capillary growth during embryonic development is driven by filopodial extensions of endothelial cells at the tips of growing capillary sprouts and that the behavior of these filopodia appears to be controlled by a VEGF gradient (12, 15). Perturbation of this gradient by altering the normal balance of VEGF isoforms caused defects in capillary growth; mice expressing only VEGF120 exhibit decreased branching and increased numbers of endothelial cells at preexisting capillary, whereas growing capillaries in VEGF188 mice are hyperbranched and have fewer endothelial cells per capillary. These observations would appear at first glance to be in conflict with our own results, that overproduction of the different isoforms induces similar effects, potentially even showing increased branching in the case of VEGF120 gene delivery. However, there are distinct differences between these situations. Our study was carried out in the adult, a state that is likely to present a very different set of responses to angiogenic stimuli compared to the developing embryo. Furthermore, in the developmental studies, VEGF120 was the only VEGF isoform present, while in our study, VEGF120 was overexpressed but the other isoforms are still present. Thus, our results in adult skeletal muscle are not inconsistent with the developmental studies. Significantly, our study is unique in that it is based on direct comparison of the three different isoforms in the same adult model system, in a context that is otherwise physiologically normal.

Similarly, a number of studies have attempted to determine the relative roles of the isoforms in tumorigenesis. For example, high intratumoral VEGF189 levels have shown a better correlation than the other isoforms with tumor angiogenesis, liver metastasis, and poor patient prognosis (49, 50). However, when tumor cells deficient in VEGF expression and tumor growth were engineered to express each isoform individually, VEGF121 and VEGF165 could rescue tumor growth, and VEGF189 could not, even though it increased vascularity (51). Furthermore, only VEGF121 and VEGF165 were up-regulated during melanoma progression (52), and VEGF121 was the most tumorigenic isoform when transfected into breast carcinomas cells (53). Even though it is tempting to construct a pattern of involvement of different isoforms in tumorigenesis, a caveat is that different isoforms have different tumorigenicity profiles when expressed in different tissues, in accordance with different VEGF receptor profiles (17).

Although excessive amounts of VEGF were studied here, it is difficult to ascribe these results to the potentially saturating amounts of VEGF being produced at the implantation sites, either by the myoblasts or by macrophages that have been shown to respond to the initial VEGF stimulus (23, 36). Indeed, if this consideration were to affect our results in any way, we would have expected the opposite effect of spreading the spatial range of VEGF’s effects farther away from the site of production. In fact, despite excessive production of VEGF, the localization of its effects was preserved. Moreover, macrophage accumulation also cannot account for the results, as we have previously reported similar gene delivery strategies that resulted in direct accumulation of macrophages that did not induce the dramatic vascular changes observed here (54). Lastly, the RT-PCR in Fig. 8 showed no significant
increase in endogenous VEGF, macrophage-derived or otherwise, further supporting the idea that it is the recombinant VEGF and not the infiltrating cells that caused the vascular growth.

Similarly, the implantation of more myoblasts producing VEGF120 than those producing the other isoforms, to compensate for the lower amount of VEGF120 being produced per cell, is a potentially confounding factor; especially given our previous observations that VEGF production per cell is more important than total average VEGF production (28). However, again the implantation of larger numbers of VEGF120-producing cells was expected to expand the range of VEGF120’s effects, which did not occur. Moreover, it was our initial expectation that the use of VEGF120 would abolish the distinction between per-cell and total VEGF amounts, and despite strong production of the protein, the striking localization of VEGF’s effect on the vasculature was not prevented, not even on a microenvironmental scale.

We have recently shown that VEGF164 microenvironmental concentration (VEGF level per cell), and not total dose, determines whether normal or aberrant angiogenesis is induced (28, 29). Although this observation goes some way toward explaining the apparent difficulty to balance safety and efficacy in VEGF gene delivery approaches to therapeutic angiogenesis (55), the use of a freely diffusible isoform such as VEGF120 could present an attractive solution to overcome the need to control the microenvironmental distribution of delivered vectors. Therefore, we sought to evaluate the effects of single-isoform overexpression in adult skeletal muscle, a therapeutically relevant tissue that is a major target of gene-delivery approaches for the treatment of ischemic conditions of the limbs. Our current study used heterogeneous populations of retrovirally transduced myoblasts, in which each cell presumably produced different amounts of VEGF isoforms depending on viral copy number and chromosomal integration site. Therefore, a limitation of this approach is that it is difficult to ascribe subtle differences, such as the trend toward increased branching in VEGF120 ears or larger hemangiomas in VEGF164 legs, to differences between isoforms rather than differences in per cell levels of VEGF. For this reason, we did not attempt to quantitate the frequency of branch points in the small regions of ear influenced by VEGF120, and we do not consider the differences in hemangioma size to be an observation of significance. Similarly, because of the highly microenvironmental nature of the effects of VEGF-expressing myoblasts on blood vessel morphology (28), measurements of total VEGF production in culture and total VEGF levels in the muscle would be of little relevance. However, the observations that the effects of the three isoforms on the vasculature were qualitatively the same and followed similar patterns of localization despite different heparin binding affinities were significant and unexpected. Our results are particularly relevant to the concept of using different VEGF isoforms for therapeutic purposes (21). Specifically, they show that a heparin-independent mechanism is responsible for tissue localization of VEGF120-induced angiogenic response; therefore, nonheparin-binding isoforms of VEGF will likely be subject to the same limitations as heparin-binding ones in the need for control over microenvironmental levels of expression.

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