Differential effects of vascular growth factors on arterial and venous angiogenesis

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Objective: Angiogenesis, the development of new blood vessels, has become an area of increased interest for both scientific and clinical application purposes. Proangiogenic agents, such as vascular endothelial growth factor (VEGF) and naltrexone, have been shown to effectively induce new blood vessel growth. Other growth factors, such as the endogenous opioid growth factor (OGF; [Met5]-enkephalin) and retinoic acid, are inhibitors of angiogenesis. The differential effects on veins and arteries, however, by any vascular growth factor, have not previously been investigated.

Methods: The chick chorioallantoic membrane (CAM) assay was used for the in vivo quantitation of angiogenesis. After 3 days of incubation, fertilized chick embryos were explanted, and a 3.2-mm methylcellulose disk containing either the known angiogenic stimulators VEGF (0.2 µg, 1.0 µg) or naltrexone (0.1 µg, 5.0 µg), or the angiogenic inhibitors OGF (1.0 µg, 5.0 µg) or retinoic acid (1.0 µg) was placed onto the CAM surface. An equal volume of distilled water served as a control. After 2 days of growth, the CAM arteries and veins were identified, and images were obtained with a digital camera. Quantitative analysis of angiogenesis was performed on a 100-mm² area surrounding the applied disk, and the number and length of the veins and arteries were measured.

Results: The angiogenic stimulators VEGF and naltrexone markedly increased both the total number and length of all blood vessels as compared with control values. The mean length of blood vessels decreased, suggesting the induction of new vessel growth. VEGF and naltrexone proportionately increased vein and arterial angiogenesis, maintaining artery/vein ratios for vessel number and length that were unchanged compared with controls. The angiogenic inhibitors, OGF and retinoic acid, notably decreased the total number and length of blood vessels in the CAM preparations. However, these compounds had a disproportionately greater inhibitory effect on arterial angiogenesis as reflected in decreased artery/vein ratios for vessel number and length.

Conclusions: The angiogenic stimulators VEGF and naltrexone induce development of veins and arteries in a proportional manner. In contrast, the angiogenic inhibitors OGF and retinoic acid demonstrated a greater inhibitory effect on arterial as compared with venous angiogenesis. Such differential effects on angiogenesis may be important in both defining mechanisms of action and designing therapeutic interventions. (J Vasc Surg 2002;35:532-8.)

Vasculogenesis and angiogenesis are complex processes. Vasculogenesis refers to the de novo development of blood vessels from mesoderm-derived angioblasts. These angioblasts differentiate into endothelial cells that arrange themselves in a tubelike fashion to form a continuous vascular network. Angiogenesis represents the budding and sprouting of new vessels from preexisting arteries and veins. Both vasculogenesis and angiogenesis involve intricate steps of proliferation, migration, and differentiation of endothelial and smooth muscle cells. They are modulated by multiple growth factors and surrounding extracellular matrix components, which influence both blood vessel growth and maturation. More recently, the opioid growth factor (OGF), [Met5]-enkephalin, has also been identified as a modulator of angiogenesis.

With respect to angiogenesis, the chick chorioallantoic membrane (CAM) has provided an in vivo model to evaluate the effects of modulators on this process. Furthermore, detailed observations of ultrastructural histodifferentiation of vessel formation can be performed on these preparations. Several investigators have attempted to quantify angiogenesis by use of the CAM model. Strick et al examined angiogenic activity by measuring vessel end point density. Other investigators attempted to quantify angiogenesis by counting the number of vessels in a mesh overlay or within an implanted gelatin sponge placed on top of the developing CAM. Kurz et al have calculated the length and distribution of...
proliferating precapillary blood vessel formation. Recently, a method to directly quantify the total number and length of blood vessels in the CAM within a defined area surrounding applied disks containing angiogenic mediators has been reported.\textsuperscript{8,13}

Arteries and veins are functionally and morphologically different. Furthermore, it has recently been shown that arterial and venous endothelial cells are molecularly distinct beginning at the earliest stages of angiogenesis.\textsuperscript{19-21} Previous studies on angiogenesis, however, have not examined the potential differential effects of angiogenic stimulators and inhibitors on the development of arteries and veins. Thus there may be different therapeutic implications for angiogenic modulators on arterial and venous diseases.

In this study we have addressed the influence of vascular growth factors on arterial and venous angiogenesis with the CAM model. The results of this investigation document for the first time differential effects by vascular growth stimulators, such as vascular endothelial growth factor (VEGF) and naltrexone, and the angiogenic inhibitors OGF ([Met\textsuperscript{5}]enkephalin) and retinoic acid, on arterial and venous growth and development.

MATERIAL AND METHODS

Chorioallantoic model. The CAM model used in these studies has been described earlier.\textsuperscript{8,13} In brief, fertilized white broiler chicken eggs were purchased from a local hatchery and maintained at room temperature for 1 day. The eggs were incubated at 37°C for 3 days and then cracked, and the embryos were explanted into spherical plastic 100-mm × 25-mm weigh boats (VWR Scientific, Bridgeport, NJ). The specimens were covered with a ster-

Fig 1. Digitized computer images of CAMs. White circles represent location of applied disks. Artery is oriented vertically with blood flowing antegrade from bottom to top of each illustration; veins have blood flowing in opposite direction. Arterial branches are indicated by arrow whereas venous branches are shown by arrowhead. A, Control specimen with venous branches intercalating between arterioles. B, Specimen exposed to VEGF shows increased vascularity. C, Treatment with opioid growth factor decreased number of arteries and veins in region of applied disk. D, Application of retinoic acid diminished growth of arteries and veins. All photographs were performed at same magnification (bar = 2 mm), with unselected variability in vessel size.
Drug delivery. Methylcellulose disks containing methylcellulose 4.5 mol/L were prepared for drug delivery as previously described. VEGF (0.2 μg, n = 10; 1.0 μg, n = 14), naltrexone (0.1 μg, n = 10; 5.0 μg, n = 11), OGF (1 μg, n = 9; 5.0 μg, n = 9), and retinoic acid (1.0 μg, n = 9) were each mixed in distilled water 10 μL. Dosages were selected on the basis of our prior work and to evaluate possible differential dose responses. Controls (n = 30, total) were performed with each series of angioactive agents and contained an equal volume of distilled water. Each of these solutions was combined with 10 μL of the methylcellulose preparation and placed on the tips of sterile 3.2-mm diameter polytetrafluoroethylene-coated rods and allowed to dry at room temperature under a vacuum hood. The dried disks were placed onto the outer third surface of the CAM, and the specimens were returned to the humidified incubator until the CAM diameter was at least 10 mm in size.

RESULTS

In comparison to controls, CAM preparations exposed to 1.0 μg of VEGF exhibited significant increases in the total number and length of all blood vessels by 95% and 25%, respectively (Figs 1, 2, and 3). This effect reflected an increased number of smaller vessels, because the mean blood vessel length of the 1.0 μg VEGF group decreased by 28% as compared with control values (Fig 4). A lower dosage of VEGF (0.2 μg) did not alter either the total length or mean length of blood vessels from control values, but the number of vessels was decreased by 20% (Figs 1, 2, and 3). This effect reflected an increased number of smaller vessels, because the mean blood vessel length of the 1.0 μg VEGF group decreased by 28% as compared with control values (Fig 4). A lower dosage of VEGF (0.2 μg) did not alter either the total length or mean length of blood vessels from control values, but the number of vessels was decreased by 20% (Figs 1, 2, and 3).
The opioid antagonist naltrexone, at a dose of 5.0 µg, significantly increased the total vessel number and length by 59% and 20%, respectively, compared with the control preparations, whereas mean vessel length decreased by 31% (Figs 2 to 4). In the group treated with 1.0 µg of naltrexone, vessel number, length, and mean vessel length were comparable with control values.

The specific effects of the proangiogenic modulators, VEGF and naltrexone, on veins and arteries were examined. In control preparations, there were twice as many arteries as veins, with a resulting artery/vein ratio of 2.0 ± 0.1, whereas total arterial length was also greater than venous length with a ratio of 1.6 ± 0.0 (Fig 1, A, Table). Treatment with 1.0 µg of VEGF increased new blood vessel growth with venous and arterial angiogenesis affected in a similar manner. The number of arteries and veins increased by 108% and 74%, respectively, whereas the lengths of arteries and veins increased by 26% and 23%, respectively, compared with control preparations. The resultant artery/vein ratios with respect to vessel number and length in the 1.0 µg VEGF group did not differ from those of control specimens (Table). The mean length of the arteries and veins also increased proportionately, by 42% and 28%, respectively, from control values, after application of 1.0 µg VEGF. The addition of 0.2 µg of VEGF induced a 30% decrease in the number of arteries and a 15% decrease in arterial length from control levels. There were no associated changes in venous growth, leading to a decrease in the artery/vein ratios as compared with control specimens (Table). Exposure to naltrexone at 5.0 µg resulted in a proportional increase in the number of arteries and veins, by 71% and 41%, respectively, from control values and an increase in the lengths of arteries and veins by 27% and 11%, respectively. No change in the artery/vein ratios with respect to vessel number was observed compared with control preparations (Table). Specimens treated with lower doses of naltrexone 1 µg did not differ in arterial and venous parameters from the control group.

In general, the angiogenic inhibitors OGF and retinoic acid appeared to have a greater effect on arterial as compared with venous angiogenesis (Table). OGF at 5 µg decreased the total number and length of all blood vessels by 31% and 19%, respectively, as compared with control values, were recorded (Figs 1, C, 2, and 3). The angiogenic inhibitor retinoic acid significantly decreased the total number and length of blood vessels by 48% and 36%, respectively, from control levels (Figs 1, D, 2, and 3). Mean blood vessel length of the retinoic acid and OGF groups were both increased by 15% from control values, indicating the presence of fewer shorter vessels (Fig 4).

In general, the angiogenic inhibitors OGF and retinoic acid appeared to have a greater effect on arterial as compared with venous angiogenesis (Table). OGF at 5 µg decreased the total number and length of arteries from control values by 34% and 22%, respectively, whereas the total number and length of veins were reduced 23% and 14%, respectively. These changes in the OGF 5 µg were specifically reflected in a reduction in the artery/vein ratio for the number of vessels (1.6 ± 0.1) and the length of vessels (1.3 ± 0.1), suggesting a greater inhibitory effect on
Differential effects of angiogenic mediators on CAM preparations

<table>
<thead>
<tr>
<th>Disk</th>
<th>Number of vessels</th>
<th>Total vessel length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Artery</td>
<td>Vein</td>
</tr>
<tr>
<td>Control (n = 30)</td>
<td>119 ± 5</td>
<td>69 ± 2</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.2 µg (n = 10)</td>
<td>83 ± 5†</td>
</tr>
<tr>
<td></td>
<td>1.0 µg (n = 14)</td>
<td>248 ± 13†</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>0.1 µg (n = 10)</td>
<td>80 ± 6</td>
</tr>
<tr>
<td></td>
<td>5.0 µg (n = 11)</td>
<td>203 ± 7*</td>
</tr>
<tr>
<td>OGF</td>
<td>1.0 µg (n = 9)</td>
<td>53 ± 5†</td>
</tr>
<tr>
<td></td>
<td>5.0 µg (n = 9)</td>
<td>79 ± 4*</td>
</tr>
<tr>
<td>Retinoic acid (n = 9)</td>
<td>60 ± 5*</td>
<td>39 ± 3*</td>
</tr>
</tbody>
</table>

Data represent mean ± SEM.
†Significantly different from controls, P < .05 by use of ANOVA with post hoc testing.
*Significantly different from group controls at P < .05 by use of a two-tailed t test.
‡Significantly different from controls, P < .005, by use of a two-tailed t test.

arteries as compared with veins (Table). The specimens treated with the 1-µg dose of OGF demonstrated a similar pattern of angiogenic inhibition to that observed with 5 µg OGF (Table). Exposure to retinoic acid also had a disproportional effect on arterial development. The number and lengths of arteries decreased by 50% and 39%, and the number and lengths of veins by 43% and 31%, respectively, compared with the control group. Treatment with retinoic acid significantly decreased associated vessel number and length artery/vein ratios in comparison to controls (Table).

DISCUSSION

The results of this study show that VEGF and naltrexone have a dose-dependent stimulatory influence on angiogenesis by use of the CAM model. In both cases, this was associated with a decrease in the mean length of both the veins and arteries, suggesting that much of this growth was due to the outgrowth of smaller branch vessels rather than merely the continued growth of arteries and veins that were already present. Indeed, a VEGF adenoviral vector was found to stimulate vessel sprouting and increased branching patterns were seen in transgenic mice with induced angiogenesis through the overexpression of fibroblast growth factor. Moreover, our data reveal that OGF and retinoic acid depress blood vessel growth. In contrast to VEGF and naltrexone, exposure to OGF or retinoic acid significantly decreased the number and length of blood vessels, with a resultant increase in the mean length of remaining vessels. The results of these experiments are consistent with those reported earlier on proangiogenic (VEGF, naltrexone) and antiangiogenic (OGF, retinoic acid) substances and show that the model system used herein was comparable to that published in previous investigations.

The question addressed in this study was what influence is exerted by angiogenic factors on arteries and veins? Our data, for the first time, indicate a differential modulation of venous and arterial angiogenesis with variable effects shown by angiogenic and antiangiogenic agents. Stimulatory agents of angiogenesis, VEGF and naltrexone, induced venous and arterial angiogenesis proportionately, maintaining the same 2:1 relative ratio of number of arteries to veins as in control preparations. In a similar manner, when the total length of arteries and veins was analyzed, increased angiogenesis by VEGF and naltrexone resulted in no significant change from the control ratio of 1.6:1. In contrast, antiangiogenic factors (OGF, retinoic acid) decreased arterial and venous angiogenesis but did so in a disproportionate fashion so that arteries were altered to a greater magnitude than veins. This was suggested by the decrease in the artery/vein ratios to 1.5:1 for the number and 1.3:1 for the length of vessels seen in these preparations. These results, on vessels functionally analogous to human pulmonary arteries and veins, would suggest that arterial development is more sensitive than veins to repressive growth factors, although the reason(s) for this are presently unclear. Greater arterial sensitivity, or arterial selectivity of these particular agents, is also suggested by the effects seen on arteries by lower doses of VEGF and OGF, when veins were not yet affected. Within the confines of the restricted number of agents and dosages used, we can propose that angiogenic modulators in general, and proangiogenic agents and antiangiogenic factors in particular, act differently on veins and arteries, but further studies are required to clarify this hypothesis.

Although relatively little is known of the process of differentiation of the vascular system, and how arteries and veins acquire their distinct structure and function, there is both an anatomic and physiological basis to explain differences in angiogenic responsivity. Recently, a molecular distinction between arterial and venous endothelial cells has been demonstrated suggesting that vessel morphogenesis is partly genetically predetermined. Wang et al. found
that ephrin-B2, a transmembrane ligand, was expressed on arterial but not venous endothelial cells from the earliest stages of angiogenesis. On the other hand, EphB4, a receptor for ephrin-B2, was observed only on venous endothelial cells. Moreover, signaling between arteries and veins appears essential for vessel morphogenesis and remodeling. Disruption of the ephrin-B2 ligand gene caused not only a defect in EphB4 receptor, thus blocking venous angiogenesis, but also a concomitant defect in the arteries. Therefore a reciprocal interaction between these two classes of vessels appears essential for angiogenesis and is codependently related at the molecular level. Adams et al demonstrated that ephrin-B2 and EphB4 have a functional and physiologically relevant interaction, which is not restricted to the arterial-venous boundary but may occur at the endothelial-mesenchymal contact zones. Others have shown a temporal relationship between changes in extracellular matrix composition and blood vessel morphogenesis. Murphy et al suggested that the sequential production of basal lamina, collagen(s), and glycosaminoglycans by the developing vessel epithelia may be critical to their final differentiation. The differentiation of arteries and veins in the chick chorioallantoic membrane correlates with the selective disappearance of hyaluronic acid and the accumulation of sulfated glycosaminoglycans in the walls of these vessels. Differences between veins and arteries are also present in other components of the vessel wall. Moessler et al have demonstrated differential expression of smooth muscle-specific protein SM 22 in arteries but not in veins. Superimposed on such anatomic differences, the arrangement of arteries and veins and their histodifferentiation may be influenced by the amount and direction of regional blood flow and pressure.

The angiogenic agents examined in these experiments are believed to act principally at the level of the endothelial cell. VEGF and its endothelial cell receptors have been studied most extensively. Mice with homozygous disruptions of VEGF receptor genes die at mid gestation from vascular structural and functional defects. A defect in the gene for VEGF receptor VEGFR-2 (flk-1) in mutant mice interfered with the differentiation of vascular endothelial cells, resulting in complete absence of organized blood vessels and embryonic death within 10 days of gestation. Disruption of another receptor gene, VEGFR-1 (flt-1), although not essential for endothelial cell differentiation, resulted in abnormal and disorganized vascular channels and early embryonic death. It has been established that the activities of OGF are receptor mediated. Previously, we had demonstrated that both OGF and its receptor were present in the endothelial cells lining the lumen of developing vessels, but it is presently unknown whether there is a differential expression of opioid receptor between arteries and veins. Although our results suggest that arterial and venous endothelial cells may respond differently to positive and negative angiogenic signals, and such expression may be dose dependent, the underlying regulating mechanisms remain to be defined. We suspect that there are multiple different pathways of activators and inhibitors of angiogenesis with a complex relationship. There appears to be codependency and reciprocal communication between arteries and veins required for vessel growth and morphogenesis. This developmental interaction may be disrupted to a greater degree with the application of antiangiogenic agents. Retinoic acid and OGF both showed greater inhibitory arterial specificity and sensitivity, although they are not known to share a common pathway of action. Restoration of vessel growth and normalization of artery/vein ratios with the OGF competitive receptor antagonist naltrexone suggest that the effect of OGF is receptor dependent, although it is not known to cross-react with any VEGF receptors.

These results are important for potential future clinical implementation. Determination of these underlying mechanisms and the vessel specificity of angiogenic modulators may lead to new approaches to therapeutic interventions directed selectively at arteries or veins. Angiogenic therapy may be used not only to assist in the development of additional collateral arterial vessels for patients with peripheral vascular or cardiac insufficiency but also to inhibit benign or malignant tumor growth, abnormal venous growth, and congenital vascular malformations.

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REFERENCES


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