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# Short Communication

## Vascular Endothelial Growth Factor, Platelet-Derived Growth Factor, and Insulin-Like Growth Factor-1 Promote Rat Aortic Angiogenesis *In Vitro*

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***The purpose of this study was to evaluate the vasoformative response of isolated vascular explants to a variety of growth factors that have been shown to stimulate angiogenesis. Rings of rat aorta were cultured in collagen gels under serum-free conditions in the presence or absence of vascular endothelial growth factor (VEGF), natural platelet-derived growth factor (PDGF), PDGF-AA, PDGF-BB, insulin-like growth factor-1 (IGF-1), transforming growth factor-α (TGF-α), transforming growth factor-β1 (TGF-β1), epidermal growth factor (EGF), interleukin-1α (IL-1α), or hepatocyte growth factor (HGF). The angiogenic response of the rat aorta was stimulated by VEGF, PDGF, PDGF-AA, PDGF-BB, and IGF-1. Maximum stimulatory effects were obtained with VEGF and PDGF-BB. By contrast, TGF-β1 and IL-1α had inhibitory activity. No significant effects were observed with TGF-α, EGF, or HGF. The vascular outgrowth of VEGF-stimulated cultures was primarily composed of microvessels, whereas that of PDGF- and IGF-1-stimulated cultures contained an increased number of fibroblast-like cells. The inability of TGF-α, TGF-β1, IL-1α, EGF, and HGF to stimulate rat aortic angiogenesis in serum-free culture suggests that either these factors require the mediatory activity of accessory cells that are not present in the rat aorta model or that blood vessels are heterogeneous in their capacity to respond to different angiogenic factors. (Am J Pathol 1994, 145:1023–1029)***

Angiogenesis, ie, the formation of new blood vessels, plays an important role in a variety of physiological processes ranging from embryonal development to the ovarian and endometrial cycles. Angiogenesis contributes also to the healing of wounds and to the progression of pathological conditions such as cancer, diabetic retinopathy, rheumatoid arthritis, and complicated atherosclerosis.[1,2] The angiogenic process is regulated by a variety of growth factors that stimulate the migration, proliferation, proteolytic activity, and organizational behavior of endothelial cells.[1] Experimental animal models and *in vitro* assays have allowed investigators to test the angiogenic activity of growth factors purified from normal and pathological tissues.[3] *In vitro* models with isolated endothelial cells have been particularly useful for studying the direct effects of angiogenic factors on endothelial cells and their mechanisms of action. These models, however, represent only a partial reconstruction of the vascular wall that *in vivo* comprises mural cells, ie, smooth muscle cells or pericytes, and fibroblasts. Because endothelial cells interact with these nonendothelial cells through paracrine mechanisms,[4] the angiogenic response of

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blood vessels to growth factors is likely to be the net result of these interactions. In fact, growth factors that stimulate endothelial cells directly may promote the growth of smooth muscle cells, pericytes, and fibroblasts, all of which can in turn produce additional angiogenic factors.[1,4–6] Furthermore, factors that do not promote endothelial growth directly may regulate angiogenesis indirectly by stimulating the nonendothelial cells of the vessel wall.[7,8] Thus, paracrine interactions between vascular endothelial and nonendothelial cells have to be taken into account when *in vitro* assays are used to test the capacity of growth factors to stimulate formation of blood vessels.

To this end *in vitro* models with intact vascular explants may reproduce more accurately the environment in which angiogenesis takes place than those with isolated endothelial cells. On this basis, we are studying angiogenesis *in vitro* using rings of rat aorta as a source of vasoformative endothelial cells.[9] We recently reported that rat aortic angiogenesis is regulated by basic fibroblast growth factor (bFGF).[10] We also suggested that factors other than bFGF might be involved in the angiogenic response of the aortic wall. The purpose of this study was to identify among a variety of growth factors implicated in angiogenesis those that stimulate the angiogenic response of the rat aorta. We report here that rat aortic angiogenesis is stimulated by vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and insulin-like growth factor-1 (IGF-1). These growth factors, which are produced by vascular endothelial and nonendothelial cells,[4,11,12] are likely to play an important role in the paracrine mechanisms that regulate angiogenesis in physiological and pathological conditions.

## Materials and Methods

### Materials

Natural PDGF, recombinant PDGF-AA and -BB, interleukin-1$\alpha$ (IL-1$\alpha$), transforming growth factor-$\alpha$ (TGF-$\alpha$), epidermal growth factor (EGF), and IGF-1 were purchased from UBI (Lake Placid, NY). VEGF and transforming growth factor-$\beta$1 (TGF-$\beta$1) were from R & D Systems (Minneapolis, MN). Hepatocyte growth factor (HGF) was obtained from Collaborative Biomedical Products (Becton Dickinson Labware, Bedford, MA). Interstitial collagen was purified from rat tail tendons.[9] MCDB 131 growth medium (endothelial basal medium) was obtained from Clonetics Corporation (San Diego, CA). Antibodies against factor VIII-related antigen (FVIII-RAg) and $\alpha$-smooth muscle actin ($\alpha$-SMA) were purchased from Dako (Carpinteria, CA) and Sigma Chemical Company (St. Louis, MO), respectively. Secondary antibodies were from Sigma. Immunoperoxidase stains were performed as described[9,10] using the avidin-biotin complex system of Vector Laboratories (Burlingame, CA).

### Rat Aorta Assay of Angiogenesis

Aortic rings obtained from 2- to 3-month-old Fischer 344 male rats were embedded in collagen gels, transferred to 16-mm wells (4-well NUNC dishes), and cultured in serum-free MCDB 131 medium at 35.5 C.[9,10] The medium (0.5 ml/well) was supplemented with growth factors at concentrations ranging from 0.1 to 50 ng/ml. Testing was conducted using triplicate cultures per dose of growth factor. Experiments were repeated to confirm positive or negative results.

### Quantitation of Angiogenesis

The angiogenic response of the rat aorta cultured with or without exogenous growth factors was quantitated by counting the number of newly formed microvessels, according to published criteria.[9] The length of the microvessels was measured by digitizing morphometry using Bioquant IV image analysis software.[13] Measurements of length were obtained from 400 to 650 microvessels per experimental group using a Leitz Laborlux microscope and a high resolution monitor. Data were analyzed with a personal computer using SPSS statistical software. The Student's *t*-test was used to evaluate the significance of differences between growth factor-treated cultures and untreated controls. Statistical significance was set at $P < 0.05$.

## Results

The effects of growth factors on the angiogenic response of the rat aorta are summarized in Figure 1. Angiogenesis was stimulated by natural PDGF, recombinant PDGF-AA and -BB, VEGF, and IGF-1 (Figures 1 and 2). By contrast TGF-$\beta$1 and IL-1$\alpha$ and antiangiogenic effects. No significant effects were seen in cultures treated with TGF-$\alpha$, EGF, and HGF.

The angiogenic effect of natural PDGF, PDGF-AA, and PDGF-BB was preceded and accompanied by an increase in the number and migratory activity of fibroblast-like cells. As a result, the front of the fibroblastic outgrowth, which in mature control cultures was behind the tips of the microvessels, advanced significantly beyond that of the vascular outgrowth (Figure 2, B–D). Concentrations of PDGF between 0.2

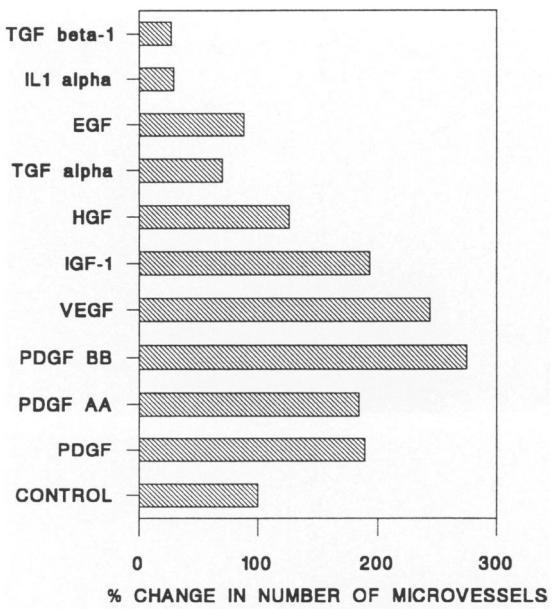


**Figure 1.** *Angiogenic response of rat aortic explants cultured under serum-free conditions with or without (control) growth factors. Shown here are the values recorded from cultures treated with the most effective concentrations of growth factors (TGF-β1, VEGF, 10 ng/ml; IL-1α, EGF, HGF, IGF-1 50 ng/ml; TGF-α, 5 ng/ml, PDGF, 2 ng/ml; PDGF-AA, PDGF-BB, 0.5 ng/ml). Angiogenesis was stimulated by IGF-1* ($P < 0.002$, $N = 6$), *VEGF* ($P < 0.001$, $N = 6$), *PDGF-BB* ($P < 0.001$, $N = 4$), *PDGF-AA* ($P < 0.007$, $N = 4$), *and natural PDGF* ($P < 0.001$, $N = 6$) *and inhibited by TGF-β1* ($P < 0.001$, $N = 6$) *and IL-1α* ($P < 0.002$, $N = 4$). *The effects of EGF, TGF-α, and HGF were not statistically significant.*

and 2.0 ng/ml stimulated angiogenesis optimally, whereas higher doses caused contraction of the collagen gel by the explant and its fibroblastic outgrowth, interfering with the angiogenic response which was delayed. This was particularly evident in cultures treated with PDGF-BB, which was the most potent of the PDGFs and produced maximum angiogenic effect at 0.5 ng/ml. Mild contraction of collagen, which did not disrupt the angiogenic response, was also observed with stimulatory concentrations of PDGFs. This resulted in the formation of radially oriented linear tracks of collagen fibers along which microvascular sprouts aligned and grew.

At variance with PDGF, VEGF produced an outgrowth composed primarily of microvessels (Figure 2E). VEGF stimulated formation and elongation of microvessels at concentrations ranging from 1 to 10 ng/ml. There was a noticeable increase in perivascular fibroblast-like cells, which was, however, minimal compared with that caused by the natural and recombinant forms of PDGF. Fibroblast-like cells in VEGF-treated cultures were seen primarily in perivascular location, suggesting that these cells were stimulated indirectly by factors produced by the endothelium.

IGF-1, like PDGF, promoted an increase in both microvessels and fibroblast-like cells (Figures 1 and 2F). Among the factors that stimulated angiogenesis, IGF-1, however, was the least potent because it required concentrations 50 to 100 times higher than those of PDGF and VEGF (50 ng/ml).

In addition to increasing the number of microvessels, PDGF, PDGF-AA, PDGF-BB, and VEGF, promoted their elongation by 70 to 80% (Figure 2). As a result, the mean length of microvessels, which in untreated control cultures was 300 μ, increased to 500 to 550 μ. Longer microvessels were also seen in cultures treated with IGF-1, whose stimulatory effect on length (30%) was less pronounced than that of the PDGFs or VEGF.

Immunohistochemical stains of growth factor-stimulated cultures and untreated controls showed that fibroblast-like cells were vimentin positive. Approximately 85 to 90% of these cells were α-SMA negative, whereas the remaining cells were α-SMA positive, as previously reported.[10] The endothelium of microvessels was vimentin positive and FVIII-RAg positive. α-SMA- and vimentin-positive periendothelial cells, consistent with pericytes, were seen around the endothelium of the newly formed microvessels.[14] Pericytes were negative for FVIII-RAg.

## Discussion

This study demonstrates that aortic explants can be used to test the angiogenic activity of growth factors *in vitro*. Using the rat aorta model we found that VEGF, PDGF, and IGF-1 promoted angiogenesis in a serum-free environment. By contrast, no significant stimulatory effects were seen with TGF-α, EGF, and HGF, whereas TGF-β1 and IL-1α had antiangiogenic activity. In a recent study we reported that bFGF promotes rat aortic angiogenesis.[10] Thus, we have now identified four growth factors—bFGF, PDGF, VEGF, and IGF-1—as important promoters of angiogenesis in serum-free vascular organ culture.

Among the factors that promote rat aortic angiogenesis, VEGF is probably the only one that acts exclusively by stimulating directly the endothelium. In fact, VEGF has been shown to stimulate proliferation and migration of endothelial cells but not of fibroblasts or smooth muscle cells.[15] Our results are consistent with this interpretation because the outgrowth in VEGF-treated cultures was primarily composed of microvessels. The mild increase in perivascular nonendothelial cells was probably the result of paracrine stimulation of these cells by the activated endothelium.[4]

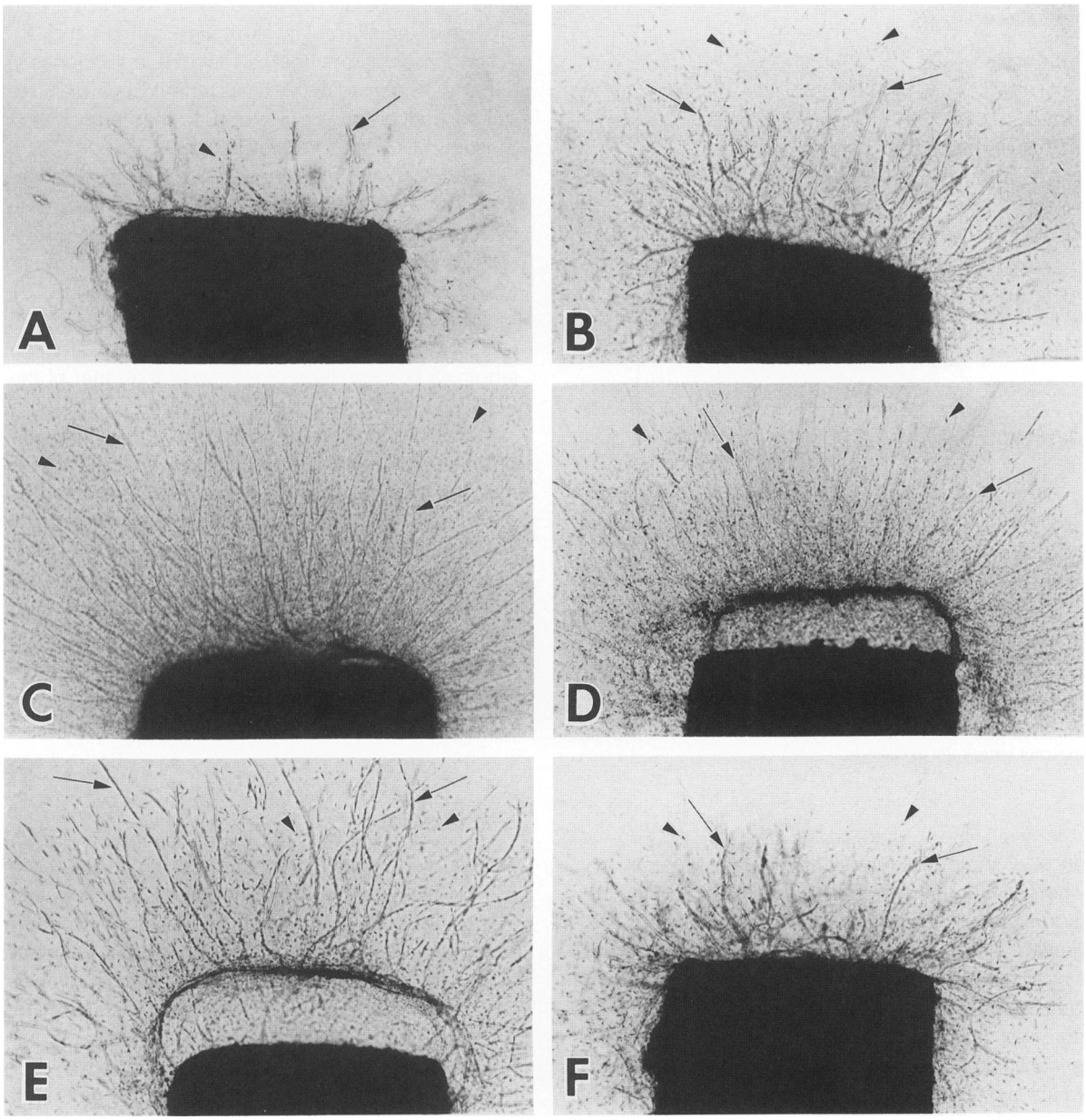


**Figure 2.** *Photomigrographs of serum-free collagen gel cultures of rat aorta. Aortic explants were cultured in the absence* (**A**) *or presence of natural PDGF* (**B**, *0.5 ng/ml*), *PDGF-BB* (**C**, *0.5 ng/ml*), *PDGF-AA* (**D**, *0.5 ng/ml*), *VEGF* (**E**, *10 ng/ml*), *and IGF-1* (**F**, *50 ng/ml*). *Microvessels are indicated by arrows, fibroblasts by arrowheads. Magnification,* ×*20.*

The angiogenic effect of PDGF suggests that rat aortic angiogenesis is regulated also by indirect mechanisms whereby fibroblasts or smooth muscle cells/pericytes produce endothelial growth factors in response to stimulation by PDGF.[5–8] In fact, large vessel endothelial cells are believed to be unresponsive to PDGF.[16] Endothelial growth factors produced by fibroblasts and smooth muscle cells include bFGF and VEGF, which have been shown to act synergistically in stimulating angiogenesis.[4,11,17,18] Additional heparin binding and non-heparin binding angiogenic factors, some of which have been partially characterized, may contribute to the mechanisms by which fibroblasts promote angiogenesis.[6,7,17,19] PDGF may also act directly because microvascular endothelial cells have been shown to express the β-subunit of the PDGF receptor and to proliferate in response to PDGF-BB.[20–22] Because the endothelium of the aortic intima in the rat aorta model switches to a microvascular phenotype,[14] we cannot rule out a transient ex-

pression by these cells of the PDGF β-receptor during the angiogenic response. This possibility is raised also by the observation that cells, which under normal conditions do not have PDGF receptors, may transiently express it during wound healing.[23] However, an indirect effect is likely to play a major role in the angiogenic activity of PDGF because rat aortic angiogenesis was stimulated also by PDGF-AA, which is unable to stimulate the proliferation of either macrovascular or microvascular endothelial cells.[16,22] The angiogenic effect of PDGF may be mediated in part by the early/immediate genes activated by this growth factor. One of these factors may be thrombospondin whose gene is activated within a few hours of exposure to PDGF.[24] Thrombospondin promotes angiogenesis in collagen gel culture of rat aorta generating fibrovascular outgrowths similar to those obtained with PDGF, suggesting a common pathway of angiogenic stimulation shared by these two factors.[7]

The contraction of the collagen gel by the aorta and its outgrowth in response to PDGF is consistent with previous reports demonstrating that PDGF is a potent vasoconstrictor.[25] Our observations also suggest that PDGF, through its capacity to induce collagen contraction by vascular outgrowths, may play an important role in the retraction of granulation tissue during wound healing. In fact, PDGF and its receptors are overexpressed by connective tissue cells and blood vessels in response to injury.[23,26] The observation that aortic cultures stimulated by PDGF generated linear tracks of collagen fibers along which endothelial sprouts aligned and grew also suggests that PDGF-mediated contraction of collagen may regulate vascular morphogenesis through mechanochemical mechanisms.[27,28] Linear tracks of collagen, which were radially oriented around the aortic explants, were produced as a result of collagen contraction by the aortic explant and its outgrowth. Although the cells responsible for the contraction of the gel are not known, it is likely that both fibroblasts and smooth muscle cells/pericytes mediated this process.[6,29] Endothelial cells may have also contributed because they have contractile properties.[6]

Of additional interest in this study was the observation that the angiogenic response of the aortic explants was promoted by IGF-1. Although IGF-1 was less potent than PDGF and VEGF, its addition to the growth medium significantly increased the number of newly formed microvessels. The lower potency of IGF-1 may be due to the endogenous production by the aortic outgrowth of IGF binding proteins that can inactivate IGF-1.[30] Our findings corroborate recent reports that have implicated IGF-1 in angiogenesis.[31,32] The observation that mouse embryos lacking the IGF-1 receptor are 45% the size of normal embryos suggests that IGF-1, which by itself is not considered an angiogenic factor, may increase the efficiency of angiogenesis during embryonal development and wound healing.[33]

The finding that TGF-β1 and IL-1α inhibit the angiogenic response of the rat aorta is consistent with previous reports that these growth factors inhibit endothelial cell migration and/or proliferation *in vitro*.[34,35] Thus, TGF-β1, which also has been shown to promote the differentiation of preexisting populations of endothelial cells,[36] behaves as an inhibitor when added to a developmental system such as the rat aorta model, which requires endothelial dedifferentiation and proliferation for a proper angiogenic response. Our results suggest that the reported *in vivo* angiogenic activity of TGF-β1 and IL-1α[37,38] is mediated by cells that are not represented in the rat aorta model. These cells may be leukocytes because angiogenic stimulation by TGF-β1 in the rabbit cornea model is preceded by an intense inflammatory reaction.[37] Furthermore, transgenic mice lacking TGF-β1 have a normal vascular system but eventually succumb to a systemic vasculitis, suggesting a major role for TGF-β1 in the regulation of the immune system.[39] It is also possible that TGF-β1 and IL-1α, because of the heterogeneity of vascular endothelial cells,[40] may have different angiogenic effects depending on the blood vessels and the animal species used to test their activity. The heterogeneity of blood vessels may also account for the inability of TGF-α, EGF, and HGF to stimulate angiogenesis in the rat aorta model, because these growth factors have been shown to be angiogenic in other models.[41,42]

In conclusion, our study demonstrates that the angiogenic response of aortic explants cultured under serum-free conditions is stimulated by VEGF, PDGF, and IGF-1. These growth factors, which are produced by vascular cells and are overexpressed, together with their receptors, in response to injury[23,26,43,44] are likely to play an important role in the autocrine/paracrine mechanisms that regulate angiogenesis during vascular wound healing. The different types of vascular outgrowths that we observed also provide an explanation for the heterogeneous stromas of neoplasms, the formation of which may depend on the production by tumor cells of different types of angiogenic factors. Thus, the vascularized fibrous stroma of breast cancer may be the result of stimulation by PDGF,[45] whereas the purely vascular stroma of renal cell carcinomas may be due to overproduction of VEGF.[46]

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