Indirect Angiogenic Cytokines Upregulate VEGF and bFGF Gene Expression in Vascular Smooth Muscle Cells, Whereas Hypoxia Upregulates VEGF Expression Only

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**Background** Hypoxia and indirect angiogenic factors may stimulate angiogenesis via induction of endothelial cell mitogen(s). To evaluate this hypothesis, we investigated whether low oxygen tension or cytokines known to promote neovascularization in vivo could modulate the expression of either vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) in human vascular smooth muscle cells (SMCs). Methods and Results SMCs were cultured with platelet-derived growth factor BB (PDGF-BB) or transforming growth factor-β1 (TGF-β1) or exposed to low oxygen tension in serum-free medium. Northern analysis detected low basal levels of VEGF and bFGF mRNA in extracts of unstimulated SMCs. However, both VEGF and bFGF transcripts increased after administration of PDGF-BB (10 or 20 ng/mL) or TGF-β1 (0.1 to 10 ng/mL). Hypoxia was a potent stimulus for VEGF gene expression but had no apparent effect on bFGF steady-state mRNA levels.

**Conclusions** These results indicate that certain indirect angiogenic cytokines, such as PDGF-BB or TGF-β1, may act via induction of bFGF and VEGF gene expression in cells resident near endothelial cells in vivo. Hypoxia constitutes a potent stimulus for VEGF gene expression but does not regulate bFGF under the same experimental conditions. (Circulation. 1994;90:649-652.)

**Key Words:** neovascularization • platelet-derived growth factor • transforming growth factor-β1 • hypoxia

The development of new blood vessels, or angiogenesis, begins with activation of parent vessel endothelial cells (ECs). At least eight polypeptide growth factors have been purified, sequenced, and shown to be responsible for normal as well as pathological angiogenesis. Growth factors shown to be mitogenic for ECs in vitro, as well as stimulating angiogenesis in vivo, have been referred to as ‘direct’ angiogenic growth factors. These include vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).

VEGF is secreted by intact cells and is mitogenic exclusively for ECs. In contrast, bFGF is neither secreted nor is its mitogenic activity limited to ECs alone; it is also a potent mitogen for vascular smooth muscle cells (SMCs), although certain data suggest that arterial injury, particularly endothelial denudation, is a prerequisite for this biological effect. The demonstrated synergism between VEGF and bFGF for induction of microvessel-like structures in two different experimental models of in vitro angiogenesis suggests that expression of these two growth factors may be concurrently regulated in vivo to optimize angiogenesis.

Certain other growth factors that stimulate angiogenesis in vivo but fail to demonstrate mitogenic activity for ECs in vitro have been called ‘indirect’ angiogenic growth factors. The basis for the disparate activities of indirect angiogenic growth factors—including platelet-derived growth factor BB (PDGF-BB) and transforming growth factor-β1 (TGF-β1)—has remained largely unresolved.

We considered the possibility that direct angiogenic growth factors might, in vivo, mediate angiogenesis induced by indirect angiogenic growth factors and thereby account for the necessary but otherwise absent mitogenic effect on parent vessel ECs and that a similar mechanism could account in part for the long-noted association between hypoxia and collateral vessel development.

Human vascular SMCs are known to produce both VEGF and bFGF. Given the resident proximity of SMCs to ECs in the vascular wall, it is possible that SMCs may constitute a local source for direct angiogenic growth factors such as VEGF and bFGF.

Accordingly, to investigate whether indirect angiogenic cytokines and/or low oxygen tension might modulate VEGF and bFGF production, we studied the effects of PDGF-BB, TGF-β1, and hypoxia on VEGF and bFGF mRNA levels in vascular SMCs. Our findings indicate that PDGF-BB and TGF-β1 induce both VEGF and bFGF gene expression. Hypoxia, in contrast, selectively increases mRNA levels for VEGF but not bFGF.

**Methods**

**Reagents** Ultrapure (>95%) platelet-derived human TGF-β1 and recombinant human PDGF-BB were generous gifts of Genzyme Corp. Phorbol-12-myristate 13-acetate (PMA) was purchased from Sigma Chemical Co.
Cell Culture

Human SMCs were cultured by explant outgrowth from unused portions of internal mammary arteries obtained at coronary bypass surgery (St Elizabeth’s Medical Center, Boston) as previously described.15 Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO) supplemented with 10% fetal calf serum (GIBCO). Confluent SMCs (passages 3 to 7) were growth-arrested in serum-free medium (IT medium)16 for 48 hours before each experiment. The cells were then treated with PDGF-BB (1, 5, 10, or 20 ng/mL) or TGF-β1 (0.1, 1, or 10 ng/mL) or exposed to hypoxia for various time intervals. Extracts of SMCs treated with PMA (20 ng/mL) for 3 hours were used as a positive control for VEGF13 and bFGF gene expression.14

Hypoxia

To achieve hypoxia, a preanalyzed air mixture (95% N2/5% CO2; Airco) was infused into an air chamber (Billups- Rothenberg) (chamber 1) or a small desiccator cabinet (Baxter) (chamber 2), constructed with inflow and outflow valves, at a flow rate of 3 L/min for 15 minutes twice a day.17 The PO2, measured with a gas analyzer (278 Blood Gas Systems, Ciba-Corning Diagnostics Corp), reached a nadir of 35 and 70 mm Hg in the culture medium in chambers 1 and 2, respectively, about 6 hours after the infusion was completed and persisted at 24 and 48 hours. SMCs in fresh IT medium were either exposed to hypoxia or maintained in normoxia (atmospheric air/5% CO2; PO2, 150 mm Hg) in the same incubator.

RNA Analysis

RNA was isolated by a phenol/chloroform extraction procedure,18 and RNA concentration was calculated from the absorbance at 260 nm. All RNA samples contained intact ribosomal RNA (rRNA) and were devoid of genomic DNA contamination, as evaluated by ethidium bromide staining of agarose gels. Twelve to 20 µg of RNA from each sample was separated by electrophoresis in 1% agarose gel containing 2.2 mol/L formaldehyde. After capillary transfer to a nylon membrane (Hybond-N, Amersham), rRNA was visualized by ethidium bromide staining to verify that equal amounts of RNA had transferred to the gel. Hybridization probes were labeled with α-32P-dCTP (New England Nuclear) with a random priming labeling kit (USB) and purified of unincorporated nucleotides with a Bio-Spin 6 minicolumn (Bio Rad). Probes had specific activities of 1×106 to 1×107 cpm/µg. The DNA probes were (1) for human VEGF, a 675-bp EcoRI/Bgl II fragment of plasmid pSVLVEGF-21 (gift of Dr N. Ferrara, Genentech Inc); (2) for human bFGF, a 0.8-kb EcoRI fragment of plasmid pHFL1-7 (gift of Dr J. Abraham, Scios Nova); and (3) for GAPDH, a 1-kb EcoRI fragment of plasmid HHCPF19 (American Type Culture Collection). Membranes were prehybridized for 1 hour and hybridized for 3 hours at 65°C in Rapid-hyb buffer (Amersham). Non-specifically bound probe was removed with a final stringency wash in 0.1× SSC+0.1% SDS at 65°C. Blots were then exposed for autoradiography at −80°C.

Results

PDGF-BB and TGF-β1 Treatment Upregulates VEGF and bFGF mRNA Species in Vascular SMCs

To investigate whether the indirect angiogenic activity of PDGF-BB and TGF-β1 documented in vivo may be explained by induction of direct EC mitogens in cells having a proximate location to ECs in vivo, we evaluated mRNA levels of VEGF and/or of bFGF in vascular SMCs after administration of TGF-β1 and PDGF-BB. Quiescent SMCs maintained in serum-free medium contained very low levels of VEGF and bFGF mRNA (Figs 1 and 2).

Administration of PDGF-BB (10 or 20 ng/mL) stimulated an increase in both VEGF and bFGF mRNA steady-state levels. Expression of both genes concurrently peaked between 3 and 6 hours. A modest increase of VEGF mRNA was still evident 7 days after stimula-
tion (Fig 1). No substantial difference was observed between treatment with 10 or 20 ng of PDGF-BB (data not shown), and no induction was observed with lower doses (1 or 5 ng/mL).

Stimulation with TGF-β1 augmented both VEGF and bFGF steady-state mRNA levels (Fig 1). Peak expression of VEGF and bFGF was observed at about 3 hours of treatment, whereas peak levels of bFGF mRNA were delayed to approximately 8 hours. At all time points evaluated, the induction of both VEGF and bFGF mRNA was dose dependent.

Hypoxia Selectively Increases VEGF but Not bFGF mRNA Species

To test whether VEGF or bFGF mediates hypoxia-induced angiogenesis, SMCs were exposed to low oxygen tension for 24 or 48 hours. Severe hypoxia (Po2, 35 mm Hg) resulted in a substantial induction of VEGF transcripts at 24 and 48 hours (Fig 2). bFGF mRNA species appeared unaffected by exposure to either severe or moderate (Po2, 70 mm Hg) hypoxia (Fig 2).

Discussion

Angiogenesis constitutes a fundamental process underlyng a variety of physiological and pathological situations. Insoluble extracellular matrix components and soluble EC mitogens coordinately modulate this complex, multifaceted phenomenon. Furthermore, some stimuli capable of inducing the development of neovessels in vivo, specifically certain cytokines and hypoxia, fail to stimulate EC proliferation in vitro, suggesting a role for additional mediators and/or cell types.

We considered whether certain so-called indirect angiogenic growth factors might stimulate vascular SMCs to express genes encoding direct EC mitogens. Such a sequential cascade would provide a mechanism by which growth factors that are otherwise nonmitogenic or frankly inhibitory for EC proliferation in vitro could instead stimulate angiogenesis in vivo. We identified two principal candidates as potential mediators of this phenomenon: VEGF, a secreted angiogenic growth factor that is exclusively mitogenic for ECs, and bFGF, a nonsecreted angiogenic polypeptide not only capable of inducing neovascularization but also known to stimulate SMC proliferation and hyperplasia in an experimental model of vascular injury. Our findings indeed suggest that indirect angiogenic cytokines upregulate expression of these two EC mitogens in vascular SMCs, whereas hypoxia stimulates VEGF expression only.

Although PDGF-BB can directly stimulate proliferation of selected populations of microvascular ECs in vitro, ECs from most vascular districts do not respond to treatment with PDGF-BB. Nonetheless, in vivo, PDGF-BB has been clearly demonstrated to induce angiogenesis in both a wound healing model and chorioallantoic membrane assay. More recently, and indirectly associated with ECs in the capillary sprouts that form during angiogenesis, express PDGF type β receptors in vivo. Furthermore, microvascular ECs isolated from rat epididymal fat pads and not directly responsive to PDGF-BB stimulation, when cocultured with myofibroblasts from the same tissue source, form capillary chords in the presence of PDGF-BB. The angiogenic action of PDGF-BB documented in this way was attributed to production of soluble EC mitogen(s) by myofibroblasts in response to stimulation with PDGF-BB.

We demonstrated that PDGF-BB treatment of human vascular SMCs concurrently induces VEGF and bFGF mRNA species. This finding indicates that PDGF-BB, a known mitogen for SMCs, could also stimulate SMCs to produce direct angiogenic factors and thereby promote angiogenesis.

The purity of mechanisms that contribute to the angiogenic activity of TGF-β1 has been the subject of intense investigation. Although clearly inhibiting growth and migration of subconfluent ECs in vitro, TGF-β1 facilitates capillary formation in vivo. Furthermore, TGF-β1 modulates the composition of the extracellular matrix and may therefore act in part by locally creating a proangiogenic environment. Although TGF-β1 directly inhibits EC proliferation, it may nevertheless direct organization of ECs into tubelike structures, depending on conditions of the culture tested. In high concentrations was found to inhibit the formation of capillary-like structures in an in vitro model of angiogenesis, whereas low doses were observed to potentiate VEGF- or bFGF-induced neovessel formation in the same assay system. This phenomenon was interpreted by Pepper et al as an example of “contextual” angiogenesis to indicate that the angiogenic response to a given cytokine is dependent on the presence and concentration of other mediators in the extracellular environment of the target ECs. Finally, Winkles and Gay also observed that TGF-β1 stimulates bFGF expression by SMCs, thus supporting the notion that direct angiogenic mediators might contribute in part to TGF-β1-induced angiogenesis. Our finding that TGF-β1 stimulation induces VEGF as well as bFGF gene expression, in concert with the above-described mechanisms, suggests that both EC mitogens are probably instrumental for mediating the indirect angiogenic effects of TGF-β1 in vivo.

In this regard, recent reports have demonstrated potent synergism between VEGF and bFGF for the development of microvessel-like structures in two different experimental models of in vivo angiogenesis. Our finding of concurrent induction of VEGF and bFGF in human vascular SMCs suggests that such synergism may effectively operate under a variety of physiological or pathological conditions characterized by increased levels of either PDGF-BB or TGF-β1. To the best of our knowledge, this is the first report of concomitant regulation of VEGF and bFGF gene expression by human vascular SMCs. This finding thus provides further support for Pepper’s concept of “contextual” angiogenesis.

Our results indicate that hypoxia is a potent inducer of VEGF but not bFGF gene expression in human SMCs. Hypoxia is generally considered to represent a fundamental stimulus for angiogenesis, although the mechanisms responsible for its angiogenic activity remain enigmatic. Increased expression of VEGF at the periphery of necrotic foci of certain neoplasms as well as hypoxia-stimulated induction of VEGF in cultured cells from glial tumor and rat skeletal muscle has been reported previously. We investigated whether, in addition to VEGF, hypoxia might also regulate bFGF gene expression and found that low O2 tension selectively modulates VEGF but not bFGF in vascular SMCs. This observation is intriguing in light of the homology between a sequence in the VEGF promoter and a nucleotide sequence in the erythropoietin
promoter identified as a binding site for a hypoxia-specific transcription factor (HIF-1). Further studies will establish whether the differential regulation of VEGF and bFGF gene expression observed in human SMCs in response to hypoxia might involve an HIF-1-controlled mechanism(s).

The present investigation sought to evaluate VEGF and bFGF gene expression in response to various indirect angiogenic stimuli and did not evaluate the corresponding levels of resulting VEGF and bFGF protein. Nonetheless, our data clearly indicate that the expression of two potent EC mitogens, VEGF and bFGF, may be coordinately regulated by certain indirect angiogenic stimuli. This phenomenon may therefore be anticipated to potentiate the angiogenic response to either PDGF-BB or TGF-β.

In contrast, hypoxia differentially regulates these EC mitogens, preferentially inducing the expression of VEGF alone.

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