Vascular endothelial growth factor B, a novel growth factor for endothelial cells

(angiogenesis/endothelium/mitosis)

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ABSTRACT We have isolated and characterized a novel growth factor for endothelial cells, vascular endothelial growth factor B (VEGF-B), with structural similarities to vascular endothelial growth factor (VEGF) and placenta growth factor. VEGF-B was particularly abundant in heart and skeletal muscle and was coexpressed with VEGF in these and other tissues. VEGF-B formed cell-surface-associated disulfide-linked homodimers and heterodimerized with VEGF when coexpressed. Conditioned medium from transfected 293EBNA cells expressing VEGF-B stimulated DNA synthesis in endothelial cells. Our results suggest that VEGF-B has a role in angiogenesis and endothelial cell growth, particularly in muscle.

Angiogenesis, the sprouting of new capillaries from preexisting blood vessels, is of central importance in many biological processes. During embryonic development, vascularization of many organs and structures is achieved by angiogenesis from the larger vascular network formed by the growth and differentiation of both early embryonic and extraembryonic splanchnic mesoderm in a process called vasculogenesis (1). Furthermore, tissue regeneration and reorganization, as well as several pathological conditions including growth and metastasis formation of solid tumors, have been shown to be dependent on the formation of new blood vessels (2). The endothelial cell is the major cell type contributing to neovascularization during angiogenesis, endothelial cells which form the inner lining of blood vessels, undergo migration with concomitant proliferation and tube formation and participate in proteolytic degradation of the basement membrane and extracellular matrix. The specific mechanisms regulating angiogenesis are not fully understood, but several potential regulators of this process have been described (3). Vascular endothelial growth factor (VEGF) is an endothelial-cell-specific mitogen and has been shown to be a potent angiogenic factor (4). It exerts its effect through two endothelial receptor tyrosine kinases (RTKs) Flk-1/KDR (5-7) and Flt-1 (8, 9), which appear to play a pivotal role in regulation of endothelial cell growth and differentiation and in maintenance of the functions of the mature endothelium (10, 11). In addition, three other RTKs have been discovered that are specifically expressed in endothelial cells, Flt-4 (12-14), TIE, and TEK (15-17), of which TIE and TEK have been shown to be indispensable for the development of a proper vasculature (18, 19). Thus, endothelial cell growth factors have several attractive features as regulators of normal and pathological angiogenesis (4, 20-22). Placenta growth factor (PIGF) (23), which is a ligand for the Flt-1 RTK (24), is structurally related to VEGF, but its biological function remains obscure at present.

Both VEGF and PGF are dimeric glycoproteins that are related in structure to platelet-derived growth factor A and B polypeptides (PDGF-A and -B), potent mitogens for smooth muscle cells, glia cells, and several other cell types (25). In this study, we have characterized a novel growth factor for endothelial cells VEGF-B.** with structural homology to VEGF, PIGF, and the two PDGF polypeptides. Our results suggest that VEGF-B plays a role in vascularization of adult and embryonic tissues, in particular of muscle.

MATERIALS AND METHODS

Cloning and cDNAs Encoding Mouse and Human VEGF-B. A cDNA clone termed pcf2, encoding part of mouse VEGF-B, was isolated from a mouse embryonic day (E) 14.5 cDNA library cloned in the yeast expression vector pPC67 (26). A 0.9-kb Sal I–Not I insert in this cDNA clone was used to screen an adult mouse heart λ ZAP II cDNA library (Stratagene). Several positive clones were subcloned by in vivo excision into pBluescript SK (+) and the nucleotide sequences of the inserts were determined (Sequenase 2.0, United States Biochemical). To obtain cDNA clones encoding human VEGF-B, 10⁶ clones of a human fibrosarcoma cDNA library HT-1080 in actt11 (Clontech) were screened with the same insert. Among several positive clones, one, termed H1.1, was analyzed and its nucleotide sequence was determined. Based on this sequence, two oligonucleotides were designed and used to amplify by reverse transcription-coupled PCR the whole coding region of human VEGF-B from oligo-(dT)-primed human erythrolemusia cell (HEL) RNA. The amplified product was cloned into the pCRII-vector of the TA cloning kit (Invitrogen) and the nucleotide sequence was determined. Standard molecular biology techniques were used throughout this work (27). The multiple amino acid sequence alignments and the phylogenetic analysis were done according to Hein (28) by using the PAM 250 distance table.

Northern Blot Analysis and in Situ Hybridization. Mouse and human multiple-tissue Northern blots (Clontech) were hybridized with a ³²P-labeled mouse VEGF-B probe (0.9-kb Sal I–Not I insert of clone pcf2). VEGF expression was analyzed with ³²P-labeled VEGF165 cDNA as the probe (a gift from Daniel Connolly) (29). The hybridizations were carried out at 42°C in 50% (vol/vol) deionized formamide/5X SSC.

Abbreviations: VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; PIGF, placenta growth factor; RTK, receptor tyrosine kinase; HUVEC, human umbilical vein endothelial cell; bFGF, basic fibroblast growth factor; E, embryonic day; BCE, bovine capillary cell.

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**The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U48800 and U48801 for mouse and human VEGF-B165, respectively).
Expression of VEGF-B and VEGF in 293EBNA Cells. cDNA inserts encoding human VEGF-B and human VEGF165 were cloned into the pREP7 expression vector (Invitrogen). Human embryonic kidney 293EBNA cells (expressing Epstein–Barr virus nuclear antigen 1) were transfected with expression plasmids by using calcium phosphate precipitation, and the cells were incubated for 48 h. Monolayers of cells were incubated in methionine- and cysteine-free medium for 30 min followed by labeling with [35S]methionine and [35S]cysteine (100 μCi/ml) (Promix, Amersham; 1 Ci = 37 GBq) in the same medium for 2 h. The labeling medium was replaced with normal medium without serum and labeled proteins were chased for 6 h. Heparin was included during the chase when indicated (100 μg/ml). Medium was collected after the chase period and cells were solubilized in 10 mM Tris-HCl, pH 7.5/50 mM NaCl/0.5% sodium deoxycholate/0.5% Nonidet P-40/0.1% SDS/aprotinin (0.1 unit/ml). Aliquots of the culture supernatants and the cell lysates were subjected to immunoprecipitation with specific antisera and analysis by SDS/PAGE. The antipeptide antiserum to human VEGF-B was generated by immunizing rabbits with a branched 23-mer oligopeptide composed of the N-terminal region (amino acid residues SQP-DAPGHQRKVVSWIDYVYTRAT). The antiserum to human VEGF was from R & D Systems.

Mitogenic Assay for VEGF-B. Conditioned medium containing human VEGF-B and human VEGF165 was collected from 293EBNA cells transfected with the appropriate expression vectors or with empty vector (mock) in the presence of heparin (1 μg/ml) 48 h after transfection. Second-passage human umbilical vein endothelial cells (HUVECs) were plated into 96-well plates (4 × 10^4 cells per well) in M-199 medium supplemented with 10% (vol/vol) fetal bovine serum and incubated for 24 h. Conditioned medium was diluted with the growth medium and cells were stimulated for 48 h. Fresh conditioned medium containing [3H]thymidine (Amersham; 10 μCi/ml) was added to the cells and stimulation was continued for another 48 h. Cells were washed with PBS and trypsinized and the incorporated radioactivity was determined by liquid scintillation counting. Bovine capillary endothelial (BCE) cells were seeded into 24-well plates and grown until confluence in minimal essential medium (MEM) supplemented with 10% fetal calf serum. Cells were starved in MEM supplemented with 3% fetal calf serum for 72 h, after which conditioned medium diluted into serum-free medium was added to the cells and the cells were stimulated for 24 h. [3H]Thymidine was included during the last 4 h of the stimulation (1 μCi/ml). Stabilities with basic fibroblast growth factor (bFGF) were carried out as above by using recombinant bFGF at 6 ng/ml (a gift from Andreas Sommer, Synergen, Boulder, CO). Cells were washed with PBS and lysed with NaOH, and incorporated radioactivity was determined by liquid scintillation counting.

RESULTS

Primary Structures of Mouse and Human VEGF-B. By using the yeast two-hybrid system for other purposes, we isolated a partial cDNA clone from a mouse E14.5 cDNA library encoding amino acid sequences similar to the VEGF family of growth factors. With this cDNA as a probe, several cDNA clones were isolated from an adult mouse heart cDNA library and from human fibrosarcoma and erythroleukemia tumor cell cDNAs. The mouse and human full-length cDNA clones encoded polypeptides of 188 amino acids containing an N-terminal hydrophilic putative signal sequence (Fig. 1). In analogy with VEGF (4), we propose that the signal peptidase cleavage site is located between Ala21 and Pro22. Hence, the processed VEGF-B polypeptides contained 167 amino acids. Mouse and human VEGF-B displayed 88% amino acid sequence identity and were highly basic, especially in their C-terminal regions. The amino acid replacements were predominantly found in the N- and C-terminal regions, while the central portions of the molecules were almost identical. Both human and mouse VEGF-B lacked the consensus sequence for N-linked glycosylation (NXT/S). Pairwise comparisons of the amino acid sequences showed that mouse VEGF-B is ~43%
identical to mouse VEGF\textsubscript{164} (21), ~30% identical to human PIGF (23), and ~20% identical to mouse PDGF-A and -B (31, 32) (Fig. 2A). The amino acid sequence motif PXC\textsubscript{4}XX\textsubscript{4}RCX\textsubscript{2}GCC, a hallmark of this family of growth factors, was also present in VEGF-B. In addition, the eight cysteine residues, involved in intra- and intermolecular disulfide bonding, are invariant among these growth factors. The C-terminal domains of mouse VEGF-B and VEGF\textsubscript{164} displayed a significant similarity with an additional eight conserved cysteine residues and stretches of basic amino acids. Phylogenetic analysis verified that VEGF-B was most related to VEGF, while PIGF and especially the two PDGF polypeptides are more distantly related (Fig. 2B).

**Tissue Expression of VEGF-B.** The expression of VEGF-B transcripts was examined in mouse and human tissue by Northern blot analysis and compared with the expression of VEGF transcripts (Fig. 3A). In mouse tissues, the most abundant expression of the 1.4-kb VEGF-B transcript was detected in heart, brain, skeletal muscle, and kidney. The major 3.7-kb VEGF transcript was expressed in heart, brain, lung, skeletal muscle, and kidney. In human tissues, the most abundant expression of the 1.4-kb VEGF-B transcript and the major 3.7-kb and 4.5-kb VEGF transcripts were detected in heart, skeletal muscle, pancreas, and prostate. Although quantitative differences exist, VEGF-B and VEGF were coexpressed in many tissues.

The distribution of VEGF-B transcripts was further examined by in situ hybridization in sections from adult mouse heart and skeletal muscle and from the early (E10) mouse embryo. In the adult heart, VEGF-B transcripts were prominently expressed in the myocardium, while no specific signal was detected in arterial smooth muscle (Fig. 3B and C). In adult striated muscle, VEGF-B transcripts were expressed by some of the myofibers whereas others seemed to lack the transcript (Fig. 3D and E). In the E10 mouse embryo, VEGF-B transcripts were detected mainly in the developing heart (Fig. 3F and G). Thus, we conclude that VEGF-B is predominantly expressed in muscular tissues and that expression can be detected at an early stage during embryonic development.

**Expression of VEGF-B in Transfected Cells.** The biochemical properties of human VEGF-B were examined in transfected human embryonic 293EBNA cells. In reducing SDS/PAGE, human VEGF-B migrated at 21 kDa (Fig. 4A). VEGF-B remained cell-associated and was not released into the culture medium unless the cells were treated with heparin (1–100 \(\mu\)g/ml) or 1.2 M NaCl (data not shown). Under nonreducing conditions, VEGF-B migrated as a dimer of 42 kDa in SDS/PAGE analysis. These results suggest that VEGF-B formed disulfide-linked dimers associated with the cell, probably through ionic interactions with extracellular or cell-surface-bound heparan sulfate proteoglycans.

The coexpression of VEGF-B and VEGF in many tissues and the ability of VEGF to form heterodimers with PIGF (33) suggested to us that VEGF-B could also form heterodimers with VEGF. To test this possibility, 293EBNA cells were cotransfected with expression vectors encoding human VEGF\textsubscript{164} and human VEGF-B. Metabolically labeled proteins were chased in the presence of heparin and immunoprecipitations were carried out with antisera to either VEGF-B or VEGF (Fig. 4B). The results show that VEGF-B can form disulfide-linked heterodimers with VEGF, which, in the absence of heparin, remain cell-associated (Fig. 5C). Interestingly, since homodimers of VEGF\textsubscript{164} are efficiently secreted into the medium (4), VEGF-B appeared to determine the release of the heterodimer.

**VEGF-B Is an Endothelial Cell Mitogen.** The ability of VEGF-B to stimulate endothelial cell proliferation was established through analysis of \(^{3}H\)thymidine incorporation in HUVECs and in BCE cells. The results show that conditioned medium from transfected 293EBNA cells expressing VEGF-B increased thymidine incorporation into DNA of both HUVECs and BCE cells (Fig. 5). As positive controls we used conditioned medium from transfected 293EBNA cells expressing VEGF and recombinant bFGF. These results demonstrate that VEGF-B is an endothelial cell growth factor.

**DISCUSSION**

Angiogenesis is a process involving several endothelial RTKs and their ligands including VEGF and PIGF (34). In this work we describe a novel growth factor for endothelial cells, VEGF-B, a nonglycosylated highly basic heparin-binding growth factor with close structural similarities to VEGF and PIGF. VEGF-B has a wide tissue distribution but is most abundant in heart, skeletal muscle, and pancreas. The expression of VEGF-B is different from that of VEGF although expression of VEGF-B and VEGF can be seen in many tissues. In situ hybridization analysis of heart and skeletal muscle identified myocytes as the principle cells expressing VEGF-B transcripts. Thus, VEGF-B, like several other growth
factors, may act in a paracrine fashion to regulate endothelial cell function.

VEGF-B forms disulfide-linked dimers that are secreted but remain bound to cells or to the extracellular matrix and can be released by heparin or high salt treatments. This association is likely to be mediated by the C-terminal basic domain, as observed for the longer and highly basic splice variants of VEGF (4, 35, 36). The association of VEGF-B to cells or to the extracellular matrix may have several important implications for regulation of its bioavailability and action in regulation of endothelial cell growth during embryonic development and in maintenance of the vasculature in adult tissues. Cell-associated VEGF-B may act as a local trophic factor and as a growth stimulus for endothelial cells by direct cell–cell interactions. This can be of particular significance in developing embryos and in contractile tissues, such as muscle, by providing spatial cues to outgrowing endothelial cells during establishment and maintenance of
the vascular tree. Alternatively, cell association may functionally inactivate VEGF-B by making it unaccessible for endothelial cells. Upon trauma or other injuries, it could be rapidly released and thus functionally activated. This latter possibility is attractive in light of the general slow turnover of endothelial cells in most tissues.

The ability of VEGF-B to heterodimerize with VEGF is consistent with the conservation of the eight cysteine residues involved in inter- and intramolecular disulfide bonding. Furthermore, the coexpression of VEGF-B and VEGF in many tissues suggests that VEGF-B–VEGF heterodimers occur naturally. The formation and cell association of such heterodimers may affect the formation of VEGF homodimers and thus indirectly control release and bioavailability of VEGF. The formation of VEGF-B–VEGF heterodimers imply that cellular signals via these two growth factors are at least partly overlapping. VEGF was recently shown also to form heterodimers with PGF (33). The formation of different heterodimeric complexes of these growth factors, their differential expression patterns during embryonic development and in adults, and different biochemical properties could provide a basis for a diverse array of regulatory signals for endothelial cells.

At least two RTKs, Flk-1/KDR and Flt-1, are involved in signaling mediated via VEGF and PGF. Whether VEGF-B binds to the same RTKs or whether a novel and yet unidentified RTK is the receptor for VEGF-B remains to be eluci-

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**Fig. 4.** Biochemical properties of VEGF-B and heterodimerization with VEGF. Human VEGF-B and human VEGF165 were expressed, either separately or in combination, in human embryonic kidney 293EBNA cells by transient transfection and the cells were metabolically labeled. Culture supernatants and detergent-solubilized cell lysates were subjected to immunoprecipitation and SDS/PAGE analysis under reducing conditions unless otherwise indicated. (A) VEGF-B was expressed as a 21-kDa protein in the transfected cells. As a control, cells were transfected with an expression vector containing the VEGF-B cDNA in reverse orientation (Rev.). By heparin treatment, VEGF-B was released from cells and found in the supernatant. Analysis of culture supernatants under nonreducing conditions (NR) showed that VEGF-B migrated as a 42-kDa species, indicating a dimeric structure. (B) VEGF-B and VEGF165 were expressed in transfected 293EBNA cells, either separately or in combination. Anti-peptide antiseras to VEGF-B or VEGF were used in immunoprecipitation analysis of culture supernatant from heparin-treated cells expressing VEGF-B, from cells coexpressing VEGF-B and VEGF165, from cells expressing VEGF165 alone, and from control cells (Rev.). Under nonreducing conditions (NR), the VEGF-B–VEGF heterodimers migrated as species of 42–46 kDa. (C) VEGF-B was expressed alone or in combination with VEGF. Culture supernatants from cells treated or untreated with heparin were subjected to immunoprecipitation with an anti-peptide antiserum to VEGF-B and analyzed by SDS/PAGE. The data show that VEGF-B homodimers and VEGF-B–VEGF heterodimers are released from cells by heparin.
FIG. 5. VEGF-B induces [3H]thymidine incorporation into endothelial cells. Conditioned medium from 293EBNA cells transfected with expression vectors for VEGF-B, VEGF165, or empty vector (mock) were diluted in respective media, applied to HUVECs and to BCE cells, and incorporation of [3H]thymidine was measured. A positive control recombinant bFGF was added to BCE cells. The columns show fold induction of [3H]thymidine incorporation compared to basal activity induced by conditioned medium from mock-transfected cells. The bars show the mean ± SD of parallel samples, and similar results were obtained in several independent experiments.

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