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26. We thank M. A. Gimbrone, Jr., R. S. Cotran, and B. Seed for helpful discussions and for critical review of this manuscript, M. Munro, G. Pinkus, and R. S. Cotran for advice on immunohistochemical staining, and E. Clark for the gift of MAb LB-2. We also acknowledge the help of K. Case and W. Atkinson in cell culture, G. Stavrakis in immunohistochemistry, and J. Kim for general laboratory assistance. The tumor cell lines SK-MEL-24, Hs 294T, HT-144, and HT-29 were obtained from the American Type Culture Collection, and murine melanoma B16-F10 cell lines were provided by B. R. Zetter and I. J. Fidler. ICAM-1 cDNA was a gift of B. Seed. Supported by National Heart, Lung, and Blood Institute grants P01 HL36028 and HL 07727.

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Vascular Endothelial Growth Factor Is a Secreted Angiogenic Mitogen

DAVID W. LEUNG, GEORGE CACHIANES, WUN-JING KUANG, DAVID V. GOEDDEL, NAPOLEONE FERRARA*

Vascular endothelial growth factor (VEGF) was purified from media conditioned by bovine pituitary folliculostellate cells (FC). VEGF is a heparin-binding growth factor specific for vascular endothelial cells that is able to induce angiogenesis in vivo. Complementary DNA clones for bovine and human VEGF were isolated from cDNA libraries prepared from FC and HL60 leukemia cells, respectively. These cDNAs encode hydrophilic proteins with sequences related to those of the A and B chains of platelet-derived growth factor. DNA sequencing suggests the existence of several molecular species of VEGF. VEGFs are secreted proteins, in contrast to other endothelial cell mitogens such as acidic or basic fibroblast growth factors and platelet-derived endothelial cell growth factor. Human 293 cells transfected with an expression vector containing a bovine or human VEGF cDNA insert secrete an endothelial cell mitogen that behaves like native VEGF.

THE ELUCIDATION OF THE FACTORS that control angiogenesis is critical for the understanding of organ development and remodeling during embryonic and fetal life, wound healing, and tissue regeneration, as well as for insight into the pathogenesis of abnormal events such as neoplastic proliferations, rheumatoid arthritis, and retinopathies (*1*).

Several factors, including epidermal growth factor, transforming growth factors α and β, tumor necrosis factor, angiogenin, and prostaglandin E_2 are angiogenic in vivo (*2*). However, these agents have little or no direct mitogenic effect on vascular endothelial cells. Their action is thought to be mediated by other angiogenic inducers, either derived from macrophages (*1, 3*) or stored in the basement membrane (*4*). In contrast, basic and acidic fibroblast growth factors (bFGF and aFGF) are very effective in inducing vascular endothelial cell proliferation in vitro and angiogenesis in vivo (*5*). In view of their wide tissue distribution, FGFs have been proposed to be major mediators of angiogenesis (*5*). However, a puzzling aspect of the two FGFs casts doubts on their role as general mediators of angiogenesis: they both lack a hydrophobic signal peptide (*6*) required for the extracellular transport according to classical secretory pathways (*7*). Accordingly, FGFs are sequestered inside the cells of origin and apparently do not have direct access to target cells (*8*). FGF may be incorporated into the basement membrane and released when specific enzymes degrade this structure (*4*). Even the newly identified platelet-derived endothelial cell growth factor (PD-ECGF) lacks a signal peptide (*9*). There is, however, strong experimental evidence that angiogenesis requires the release of diffusible factors (*10*). Furthermore, media conditioned by a variety of transformed and untransformed cells exert mitogenic activity on endothelial cells (*11*), suggesting the secretion of mitogens distinct from FGF.

We identified and purified a heparin-binding vascular endothelial growth factor (VEGF) from media conditioned by bovine pituitary follicular or folliculostellate cells (FC) (*12*). This growth factor is a dimeric protein with a molecular mass of 45,000, composed of two subunits of identical molecular mass (23,000) and has a unique NH_2-terminal amino acid sequence. VEGF is a potent mitogen for vascular endothelial cells isolated from both small and large vessels, but does not affect the growth of BHK-21 fibroblasts, lens epithelial cells, corneal endothelial cells, keratinocytes, or adrenal cortex cells. The presence of VEGF in high concentrations in the medium conditioned by FC suggested that VEGF is a secreted molecule and that it may be a soluble mediator of endothelial cell growth and angiogenesis.

We isolated cDNA clones that encode bovine VEGF by screening a cDNA library (*13*) prepared from FC (*14*). This was done with a 59-base nucleotide probe (*15*) based on the NH_2-terminal amino acid sequence (Fig. 1A, position 2 to 21) of VEGF. Twenty hybridizing clones were identified in a library of 1.5×10^6 clones and two of these were sequenced for all the coding and for much of the noncoding regions. Their sequences were identical. The complete cDNA and corresponding protein sequence of one VEGF clone, bVEGF.6, is shown in Fig. 1A. This bovine VEGF cDNA clone contains an open reading frame of 190 amino acids. The NH_2-terminal amino acid sequence determined from the purified native bovine VEGF is preceded by 26 amino acids beginning with a methionine. The DNA sequence CCGAAACC preceding the ATG codon encoding the methionine agrees well with the initiation site consensus sequence (GCC)GCC$^{A}_{G}$CCATG in vertebrates (*16*). These 26 residues contain a hydrophobic core of 16 amino acids flanked by polar or charged residues indicative of a secretory signal sequence (*7*). The amino acid sequence Ser-Gln-Ala at positions −3 to −1

D. W. Leung, G. Cachianes, W.-J. Kuang, D. V. Goeddel, Department of Molecular Biology, Genentech, South San Francisco, CA 94080.
N. Ferrara, Department of Developmental Biology, Genentech, South San Francisco, CA 94080.

*To whom correspondence should be addressed.

(Fig. 1) follows the rule for a signal sequence cleavage site (7). Thus the mature bovine VEGF monomer is expected to have 164 amino acids with a molecular mass of 19,162. The presence of a potential N-linked glycosylation site (17) at Asn^{74} suggests that VEGF is a glycoprotein. Clusters of basic amino acids present at the positions around 110, 123, and 163 could be responsible for the binding of the molecule to heparin, by analogy with what has been proposed for bFGF and aFGF (6). The 3′ untranslated region is 421 base pairs long. The polyadenylation [poly(A)] sequence AATAAA (18) is found 20 base pairs from the poly(A) addition site.

RNA (Northern) blotting experiments were performed with poly(A)$^+$ RNA prepared from FC. A single hybridization band having a size of 3.7 kb was detected (Fig. 2). VEGF mRNA is expected to have a 5′ noncoding region that exceeds 2.5 kb, since the distance between the initiator ATG codon and the polyadenylation site is less than 1 kb (Fig. 1). Examination of the 545 bp of 5′ noncoding sequence from clone VEGF.6 (Fig. 1A) shows that this region has a GC content that exceeds 75%. This region contains an AUG codon at position 360 and a stop codon three codons downstream. It is not known whether there are other AUG codons farther upstream in the 5′ noncoding sequence and how this exceptionally long leader sequence would affect gene expression in vivo (16).

We screened several human cDNA libraries with bovine cDNA clone as a probe. Human VEGF cDNA clones were identified in a library prepared from phorbol ester–activated HL60 promyelocytic leukemia cells (19). The complete cDNA and translated protein sequence of a human clone hVEGF.21 are shown in Fig. 1B. The overall amino acid homology between human and bovine VEGF sequences exceeds 95%. Human VEGF is expected to have an additional amino acid (165) because of the insertion of a Gly in position 6 (Fig. 3). We also identified human clones encoding for a shorter and a longer molecular species of VEGF, which display a deletion of 44 amino acids between position 116 and 159 and an insertion of 24 amino acids (KSVRGKGKGQKRKRKKSRYKSWSV) in position 116, respectively. Therefore, the mature proteins are expected to have 121 and 189 amino acids, respectively. In both cases

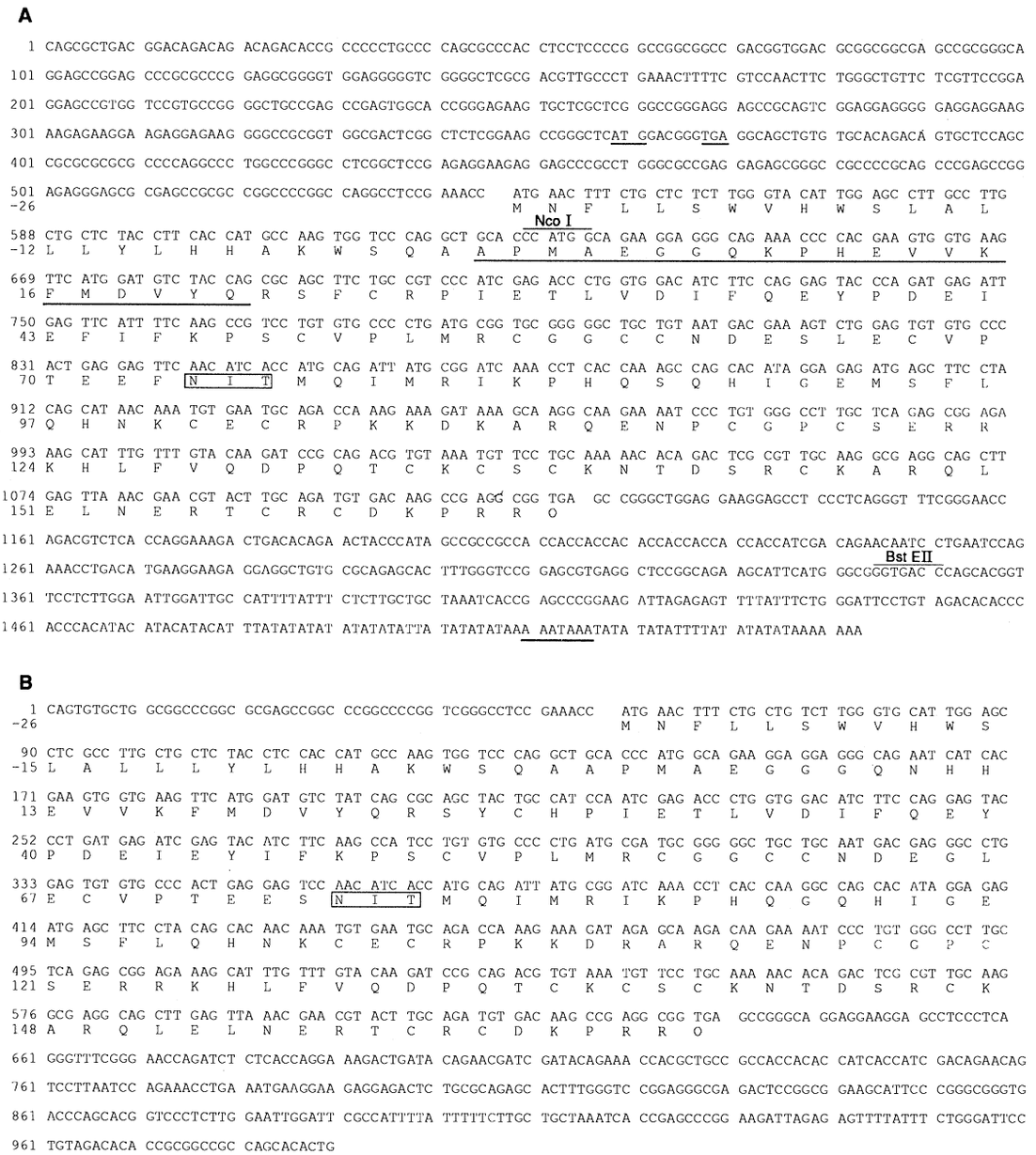

Fig. 1. Nucleotide sequence and deduced amino acid sequence of a bovine (**A**) and a human (**B**) VEGF cDNA clone. The amino acid sequence derived from NH_2-terminal sequence analysis is underlined. The protein sequence is numbered starting with 1 at the mature NH_2-terminal alanine. The putative glycosylation site is boxed. ATG and stop codons found in the 5′ noncoding region and the poly(A) signal AAATAAA are also underlined. DNA sequence analysis was performed by the dideoxy chain termination method with cDNA fragments subcloned into plasmid vectors (25). Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Asn^{115} is replaced by a Lys. This suggests the existence of several types of homodimers and, potentially, also of heterodimers of VEGF. The most likely explanation for this molecular heterogeneity is alternative splicing of mRNA. Whether these various molecular species have different biological activities remains to be established.

A search of several databases indicated significant homologies between the amino acid sequence of VEGF, the sequences of the A and B chains of platelet-derived growth factor (PDGF) (Fig. 3), and the product of the *sis* oncogene (*20*). All eight cysteine residues found in the A and B chains of PDGF are conserved in VEGF. However, VEGF contains eight additional cysteines within its COOH-terminal 50 amino acids. PDGF is active on a wide variety of cells types of mesenchymal origin and inactive on endothelial cells (*20*), whereas VEGF appears to be a highly specialized molecule selective for vascular endothelial cells.

To confirm the authenticity of the VEGF cDNA clones, we assembled cDNA inserts for bovine and human VEGF in a mammalian expression vector containing a cytomegalovirus (CMV) promoter and other features for high-level expression in mammalian cells (*21*). The media conditioned by human 293 cells transfected with the vector pbVEGF.6 and phVEGF.21 containing, respectively, the bovine and human VEGF cDNA inserts, promote the proliferation of capillary endothelial cells (Fig. 4). In contrast, the media conditioned by untransfected human 293 cells or cells transfected with the vector alone had essentially no mitogenic activity on this cell type.

The verification of the hypothesis that VEGF may have a role in the regulation of blood vessel proliferation requires the demonstration that it is capable of inducing angiogenesis in vivo (*1*). We found that native FC-derived VEGF is able to induce a marked angiogenic response in the chick chorioallantoic membrane, in the absence of significant inflammation (*22*).

The presence of VEGF in FC suggests that these cells, besides being involved in ion transport (*14*) or in the paracrine regulation of pituitary hormone secretion (*23*), have a role in the development and maintenance of the pituitary portal vessels. Furthermore, factors derived from FC may be responsible for the arteriogenesis that accompanies the growth of estrogen-induced pituitary tumors in the rat (*24*). Such induction of arterial vessel growth may, at least in part, be mediated by VEGF. Also, the presence of

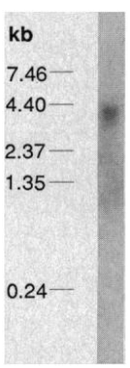

Fig. 2. Northern blot analysis of bovine pituitary FC mRNA. FC poly(A)$^+$ RNA (5 μg) and RNA markers were separated by electrophoresis in a 1% agarose gel containing 2.2*M* formaldehyde, blotted to nitrocellulose, and hybridized under stringent condition (*26*) to a ^{32}P-labeled probe (*27*) prepared from a 720-bp Nco I–Bst EII fragment from bovine VEGF.6 cDNA clone.

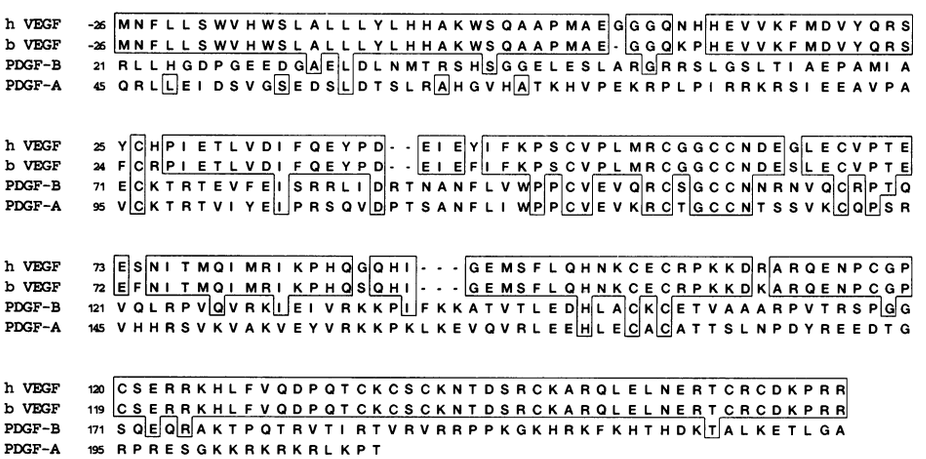

Fig. 3. Comparison of the bovine and human VEGF amino acids sequences with those of A and B chains of human PDGF. Only part of the sequences of PDGF are shown, and the similar sequences are boxed. The mature A and B chains of PDGF start at positions 87 and 82, respectively (*20*). Abbreviations for the amino acid residues are as in the legend to Fig. 1.

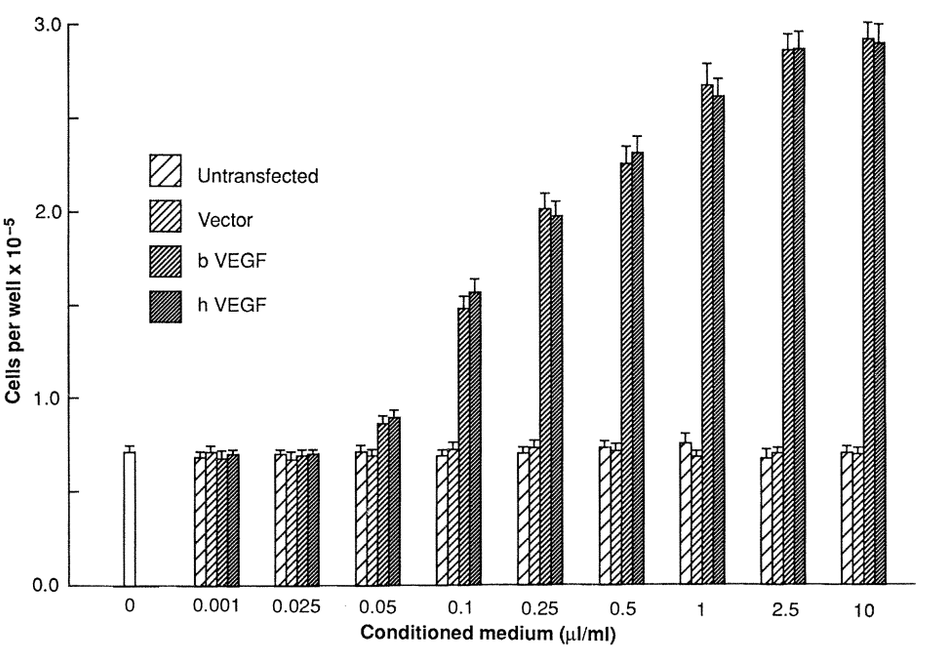

Fig. 4. Transient expression of bovine and human VEGF in mammalian cells. Subconfluent human 293 cells, cultured in the presence of Dulbecco's modified Eagle's medium/F12 (1:1) supplemented with 10% fetal bovine serum and 2 m*M* glutamine were transfected by the calcium phosphate coprecipitation method (*28*) with a vector containing the bovine (b) or the human (h) VEGF cDNA insert (*25*) or with vector alone. The human clone hVEGF.21 encodes the 165–amino acids species of VEGF (Fig. 1B). After overnight incubation, the media were removed and replaced with fresh growth media. After 48 hours the media were collected. Conditioned media from untransfected cells, cells transfected with the vector alone, and cells transfected with the vector containing the VEGF inserts were tested for mitogenic activity on capillary endothelial cells from bovine adrenal cortex, as previously described (*12*). The indicated portions of conditioned medium were added to cells, which were dissociated by exposure to trypsin after 5 days and counted in a Coulter counter. Values are means ± SEM of triplicate determinations.

cDNA clones encoding VEGF in a library prepared from differentiated HL60 cells suggests that the growth factor is produced by monocytes and macrophages (*19*). This localization would be interesting in view of the pivotal role these cells have in physiological and pathological angiogenesis and in tissue repair (*1*). The availability of cDNA clones and specific antibodies should make it possible to address these questions, as well as the more general question of the distribution of VEGF in normal versus malignant tissues and its significance and function in vivo.

Note added in proof: After this work was reviewed for publication, we were informed that Keck *et al.* (*29*) simultaneously reported the cloning of vascular permeability factor (VPF). Apparently, VPF and VEGF have similar amino acid sequences.

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21. A mammalian expression vector pRK.CXRHN, a derivative of pRK5 (constructed by R. Klein and D. V. Goeddel) with a different polylinker sequence was used. The Eco RI inserts containing, respectively, the full-length VEGF cDNA from λbVEGF.6 and λhVEGF.21 were subcloned into the polylinker region of pRK.CXHRN in such a way that the transcription of the VEGF cDNA is directed by the CMV promoter. These plasmids, pbVEGF.6 and p.h.VEGF.21, were used for subsequent transfection experiments.
22. The chorioallantoic membrane was dislocated by the false air sac technique as previously described [V. Hamburgher, *A Manual of Experimental Embryology* (Univ. of Chicago Press, Chicago, IL, 1942]. A window of 2 cm^2 was cut into the eggshell of 8-day-old fertilized eggs. Native FC-derived VEGF was dried and resuspended in vehicle consisting of phosphate-buffered saline containing Sephadex G50 beads (1 mg/ml). Vehicle or VEGF (50 ng) was applied in 10-μl aliquots to the chorioallantoic membrane. After 72 hours, the neovascular response was evaluated as previously described [R. Phillis and S. Kumar, *Int. J. Cancer* **23**, 82 (1979)]. VEGF induced a positive angiogenic response in 85% of embryos (n = 59). Vehicle induced a positive response in 10% of embryos (n = 50).
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30. We thank K. Fisher for making available to us the cDNA library prepared from HL60 cells. We also thank J. Winer for technical assistance.

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Vascular Permeability Factor, an Endothelial Cell Mitogen Related to PDGF

PAMELA J. KECK, SCOTT D. HAUSER, GWEN KRIVI, KIM SANZO, THOMAS WARREN, JOSEPH FEDER, DANIEL T. CONNOLLY*

Vascular permeability factor (VPF) is a 40-kilodalton disulfide-linked dimeric glycoprotein that is active in increasing blood vessel permeability, endothelial cell growth, and angiogenesis. These properties suggest that the expression of VPF by tumor cells could contribute to the increased neovascularization and vessel permeability that are associated with tumor vasculature. The cDNA sequence of VPF from human U937 cells was shown to code for a 189–amino acid polypeptide that is similar in structure to the B chain of platelet-derived growth factor (PDGF-B) and other PDGF-B–related proteins. The overall identity with PDGF-B is 18%. However, all eight of the cysteines in PDGF-B were found to be conserved in human VPF, an indication that the folding of the two proteins is probably similar. Clusters of basic amino acids in the COOH-terminal halves of human VPF and PDGF-B are also prevalent. Thus, VPF appears to be related to the PDGF/v-*sis* family of proteins.

VPF WAS FIRST IDENTIFIED IN RODENT tumor cell lines ((*1–3*), but has recently been purified to homogeneity from the serum-free conditioned medium of the human histiocytic lymphoma cell line U937 (*4*). Amino acid sequencing of the NH_2-terminus of guinea pig VPF (gVPF) or the NH_2-terminus and tryptic peptides of human VPF (hVPF) did not reveal, at the time, obvious similarities to

P. J. Keck, J. Feder, D. T. Connolly, Department of Cell Culture and Biochemistry, Monsanto Company, St. Louis, MO 63167.
S. D. Hauser, G. Krivi, K. Sanzo, T. Warren, Monsanto Company, Department of Biological Sciences, St. Louis, MO 63198.

*To whom correspondence should be addressed.