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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13. Messenger RNA was prepared from bovine pituitary follicular cells by the guanidine thiocyanate–LiCl method [G. Cathala *et al.*, *DNA* **2**, 329 (1983)] followed by oligo(dT) cellulose chromatography [H. Aviv and P. Leder, *J. Mol. Biol.* **134**, 743 (1972)]. Oligo(dT) and random primed cDNAs were prepared with a cDNA synthesis kit (Amersham). The resulting double-stranded DNAs were ligated to hemikinased Eco RI adaptors [D. W. Leung, *et al.*, *Nature* **330**, 537 (1987)]. The cDNAs with Eco RI cohesive ends were purified by spin dialysis through Sephacryl S500HR before being cloned into λgt10 vectors for the generation of cDNA libraries. These libraries were screened with the probe 5′CCTATGGCTGAAGGCGGCCAGAAGCCTCACGAAGTGGTGAAGTTCATGGACGTGTATCA, a 59-base synthetic probe based on the 20 amino acids sequence found at the NH_2-terminal region of VEGF. Hybridization of the probe labeled at its 5′ end with ^{32}P was done under nonstringent condition (*24*) without any dextran sulfate and the filters were washed in 0.15*M* NaCl, 15 m*M* sodium citrate, and 0.1% SDS at 50°C.
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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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Vascular Permeability Factor, an Endothelial Cell Mitogen Related to PDGF

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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

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other known proteins. Oligonucleotide probes based on these amino acid sequences were used to screen a cDNA library prepared from phorbol ester–stimulated U937 cells. The largest of the seven clones isolated contained a 3.5-kb insert. This probably represents a full-length cDNA since the size of the mRNA was approximately 3.8 kb by Northern blot analysis.

The partial sequence of the cDNA from the 3.5-kb insert and the sequence of the corresponding predicted amino acids of hVPF are shown in Fig. 1. The cDNA sequence contains an open reading frame that codes for a 215–amino acid protein. The methionine codon at position −26 probably represents the start of translation. The alanine at position 1 is the first amino acid detected by NH_2-terminal amino acid sequencing of U937-derived hVPF. The intervening 26–amino acid hydrophobic sequence (61% nonpolar residues) closely resembles the classical consensus signal sequence observed for other secreted proteins (*5*) and is evidently cleaved to generate mature VPF. This is consistent with observations that VPF is a secreted protein. An in-frame stop codon is found at amino acid position 190. A GC-rich area of nucleotides was found upstream from the methionine start codon (nucleotides 1 to 156) and may represent untranslated sequences similar to those observed in cDNAs of other factors including platelet-derived growth factor B (PDGF-B), PDGF-A, transforming growth factor–β (TGF-β), insulin-like growth factor II, basic fibroblast growth factor (bFGF), and c-*myc* cDNAs (*6–11*).

The predicted amino acid sequences for positions 1 to 18, 17 to 23, 33 to 45, and 46 to 55 correspond exactly with those obtained by amino acid sequencing of the NH_2-terminus and of three different tryptic peptides, respectively (*4*). The predicted molecular mass of 25,384 daltons for a single monomer of the 189–amino acid, nonglycosylated hVPF is slightly larger than the estimated sizes of the reduced subunits of VPF observed by SDS–polyacrylamide gel electrophoresis (PAGE), which vary between 18 to 24 kD. This heterogeneity may be a result of proteolytic processing of the COOH-terminus, which is extremely rich in basic amino acids. Multiple bands could also arise from differential glycosylation since a single N-linked glycosylation site was identified at amino acid position 75 (Fig. 1).

The NH_2-terminal region of the predicted amino acid sequence of U937 hVPF was compared with that of gVPF isolated from guinea pig line 10 tumor cells (*2, 3*) (Fig. 2). The two sequences are 78% identical in this region. Antibodies directed to peptides at the NH_2-terminus of gVPF adsorbed out the permeability-enhancing and growth-promoting activities of both gVPF (*2, 3*) and hVPF (*4*). The same antibodies recognized all of the heterogeneous forms of gVPF observed in immunoblots (*3*). These results, in addition to the identity with the hVPF amino acid sequences cited above, provide evidence that this cDNA corresponds to hVPF.

This identification was further verified by expression of the VPF protein in heterologous cells. The hVPF cDNA was inserted into a bovine papilloma virus (BPV)–based expression vector, and the resulting plasmid was transfected into mouse C127 cells. Conditioned media from stable transfectants were injected intradermally into a guinea pig to assay for the presence of permeability-

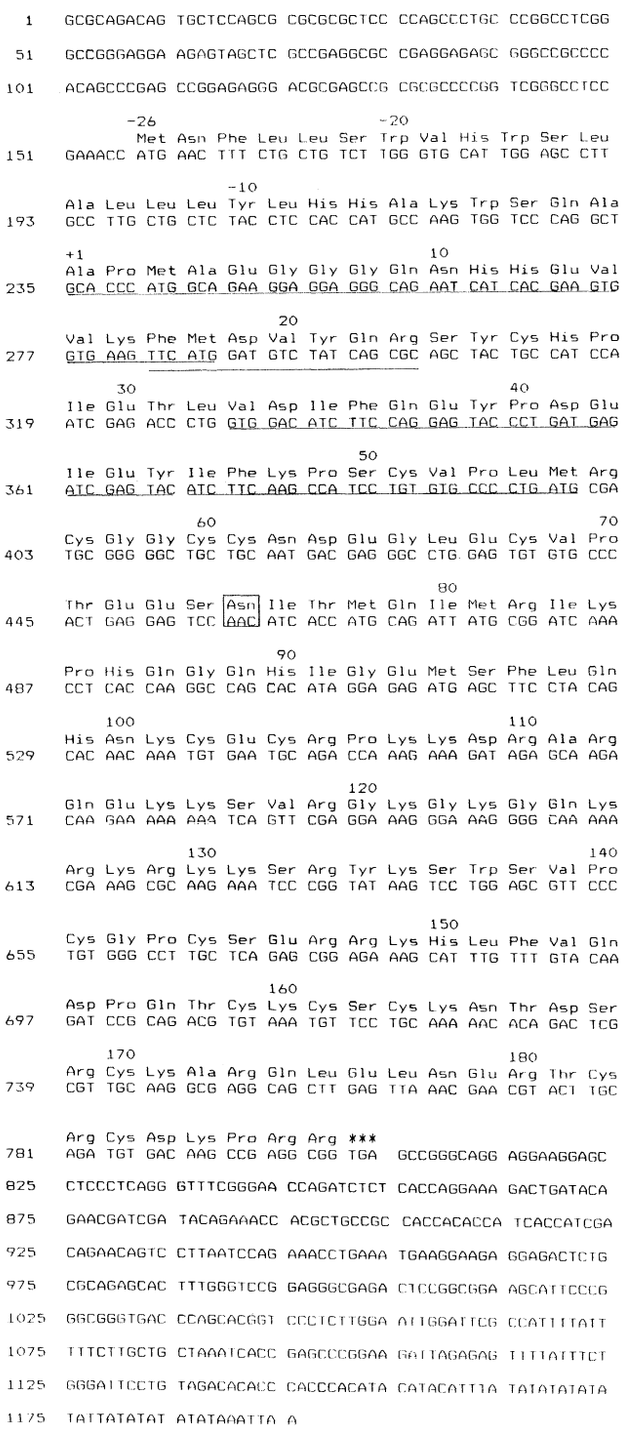

Fig. 1. The cDNA sequence and predicted amino acid sequence of hVPF. Nucleotide numbering starts with 1, even though approximately 1300 bp and 1600 bp of unsequenced cDNA flank the coding region on the 5′ and 3′ ends, respectively. The putative amino acid signal sequence is designated as amino acids −26 to −1, whereas mature hVPF is numbered 1 to 189. The predicted amino acid sequences from the cDNA clone matched amino acid sequences previously determined (*4*) and are underlined. A single potential N-glycosylation site is boxed. Approximately 5×10^5 clones from a λgt10 cDNA library constructed from phorbol ester–stimulated U937 cells (Clontech) were screened with two oligonucleotide probes. One probe, 5′-GTTCTGGCCGCCG-CCCTCGGCCATAGGA-GC-3′, which was based on NH_2-terminal amino acid sequencing, hybridized to a single λgt10 clone. A second probe, 5′-GTGGACA-TCTTCCAGGAGTACC-CCGACGAGATCGAGT-AC-3′, based on sequence information from a tryptic peptide corresponding to amino acids 33 to 45 hybridized to seven clones containing inserts ranging in size from 0.8 to 3.5 kb. The screening procedure was that of Ullrich (*30*) with the following modifications: purified tRNA (0.1 mg/ml) was used instead of salmon sperm DNA, and the sodium pyrophosphate and adenosine triphosphate were deleted from the hybridization solution. The largest clone, 3.5 kb, was subcloned into pUC9 (New England Biolabs) and 1195 bp of sequence was obtained by the dideoxy-termination method, with reagents obtained from United States Biochemicals. The sequence corresponding to nucleotides 288 to 1110 was obtained by sequencing both strands of DNA. Nucleotides 147 to 288 were determined from one strand, but were sequenced twice with two different sequencing primers.

enhancing activity. Significant amounts of VPF activity were expressed by cells containing the hVPF construct, but not by control cells transfected with the vector alone (Fig. 3). In other experiments, the activity could be completely immunoadsorbed out with an immunoglobulin G directed against gVPF. These results demonstrated that the cDNA was correctly identified as hVPF and that this gene alone is sufficient for expression of VPF activity.

Comparisons of the hVPF nucleotide sequence with other known nucleotide sequences in the European Molecular Biology Laboratory (EMBL) (*12*) and GenBank (*13*) databases revealed low but significant homology of hVPF with PDGF-B, PDGF-A (Fig. 4), and other related members of the PDGF/v-*sis* oncogene family of proteins, but not with other known sequences including aFGF, bFGF, TGF-β, epidermal growth factor, or platelet-derived endothelial cell growth factor (*14*). The identities of the deduced amino acid sequence of hVPF with PDGF-B and PDGF-A are 18% and 15%, respectively. In contrast, 44% of the amino acids of PDGF-B and PDGF-A are identical. All eight cysteines in the PDGF-like proteins are conserved in hVPF, thereby suggesting that these proteins have similar tertiary structures in this region. In addition, hVPF contains eight other cysteines in the COOH-terminal portion of the molecule, all of which are outside the cysteine-rich domains of PDGF-B and PDGF-A. Another major structural feature that hVPF and the PDGF proteins share is the presence of several lysine- and arginine-rich regions in the COOH-terminal halves of the polypeptide chains.

Both PDGF (*15, 16*) and hVPF (*4, 17*) are highly basic dimeric proteins that are stable to boiling and to acid treatment, but labile to treatment with sulfhydryl reducing agents. In addition, both hVPF (*4*) and PDGF (*16*) display microheterogeneity with respect to size and charge in SDS-PAGE and other electrophoretic systems. It has been proposed that the heterogeneity of PDGF-A is due to proteolytic processing in the highly basic COOH-terminal region of the protein (*7, 15*). Human VPF also contains a large number of lysine and arginine residues (31 of 89, 35%) in its COOH-terminal region that could serve as substrates for serine proteases. This suggests that COOH-terminal proteolysis could contribute to the heterogeneity of VPF.

PDGF and gVPF are structurally related and are both potent mitogens, but their target cell specificities and biological properties are different. PDGF stimulates in vitro growth of fibroblasts and smooth muscle cells (*15, 18*), whereas VPF stimulates endothelial cell proliferation (*2*). Furthermore, PDGF does not enhance permeability when tested in the Miles permeability assay (*2, 19*).

Although the normal physiological function of PDGF is thought to involve wound healing, the v-*sis* oncogene from simian sarcoma virus contains the sequence for PDGF-B (*20, 21*). Expression of the v-*sis* protein leads to transformation and tumorigenicity (*22, 23*). The permeability-enhancing activity of VPF, as well as its mitogenic and angiogenic properties suggest that it too could be a wound-healing agent if expressed under appropriate conditions. The same properties suggest that the expression of VPF by tumors could lead to leakiness of tumor-associated vasculature (*24–27*) and to tumor angiogenesis (*28*). VPFs that are immunologically related to gVPF are produced by various human and rodent tumor cell lines (*29*) and have been purified from human U937 tumor cells (*4*) and guinea pig line 10 tumor cells (*2, 3*). It is possible that VPF, as described here, could itself be an oncogene product that is related to a non–tumor-associated VPF-like protein yet to be discovered. This would be analogous to the relation between v-*sis* and PDGF-B. The description of the VPF sequence should allow for the design of suitable probes to examine this possibility and to determine the normal function of VPF and related proteins.

Note added in proof: After completion of this work, Ferrara and Henzel (*32*) described a protein called bovine vascular endothelial cell growth factor (VEGF). The

```
                        5                  10                  15
hVPF  Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu Val Val
      *   *   *   *   *           *                   *   *   *
gVPF  Ala Pro Met Ala Glu Gly Glu --- Gln Lys Pro Arg Glu Val Val
                        5                  9                   14

                       20                  25                  30
hVPF  Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro Ile Glu
      *   *   *   *   *   *       *   *   *   *   *   *   *   *
gVPF  Lys Phe Met Asp Val Tyr Lys Arg Ser Tyr Cys Arg Pro Ile Glu
                       19                  24                  29

                       35
hVPF  Thr Leu Val Asp Ile Phe Gln
      *   *   *   *   *   *
gVPF  Met Leu Val Asp Ile Phe Gln
                       34
```

Fig. 2. Comparison of the NH_2-terminal sequences of hVPF and gVPF. The NH_2-terminal sequence of U937 cell hVPF is taken from Fig. 1, and the sequence of gVPF was determined by amino acid sequencing of purified gVPF from guinea pig line 10 tumor cells (*2, 3*).

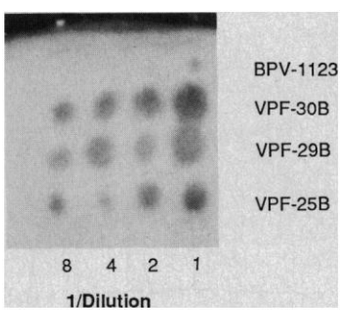

Fig. 3. Expression of permeability-enhancing activity by C127 cells transfected with the hVPF gene. Serum-free conditioned medium was collected from three selected cell lines (VPF-25B, VPF-29B, and VPF-30B) transfected with a BPV expression vector containing the hVPF cDNA sequence or from a control line (BPV-1123) transfected with the BPV vector only. Medium was concentrated fivefold with a Centricon-10 ultrafiltration device before the Miles permeability assay was performed (*31*). A positive response is indicated by the appearance of a spot at the site of intradermal injection of sample into the guinea pig, indicating extravasation of Evan's blue dye from the circulation. The photograph showing the back of the guinea pig was taken approximately 15 min after the intradermal injection of samples. The hVPF cDNA was expressed in mammalian cells with a BPV vector. This vector is based on the 100% viral genome and utilizes the mouse metallothionein I promoter and the SV40 late polyadenylate [poly(A)] addition site to regulate the expression of foreign genes. The vector was linearized at the single Bam HI site between the promoter and poly(A) addition site. The 5′ overhanging ends of the vector fragment were filled in with Klenow enzyme and dNTPs. Similarly, the plasmid containing the hVPF cDNA was digested with Xma I and the 1021-bp VPF fragment was isolated by gel electrophoresis. This blunt-end fragment was then inserted into the vector by ligation. Mouse C127 cells were cotransfected with the hVPF expression vector and pSV2neo by standard techniques and G418-resistant transfectants were selected. Colonies were picked and expanded into stable lines for assay.

```
                    .          20         .          40         .
hVPF   APMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPD--EIEYIFKPSCVP
PDGF-B            SLGSLTIAEPAMIAECKTRTEVFEISRRLIDRTNANFLVWPPCVE
PDGF-A              SIEEAVPAVCKTRTVIYEIPRSQVDPTSANFLIWPPCVE

          60         .          80         .          100
hVPF   LMRCGGCCNDEGLECVPTEESNITMQIMRIKPHQGQHI---GEMSFLQHNKCECR
PDGF-B VQRCSGCCNNRNVQCRPTQVQLRPVQVRKIEIVRKKPIFKKATVTLEDHLACKCE
PDGF-A VKRCTGCCNTSSVKCQPSRVHHRSVKVAKVEYVRKKPKLKEVQVRLEEHLECACA
           *
                    .         120         .         140         .         160
hVPF   PKKDRARQEKKSVRGKGKGQKRKRKKSRYKSWSVPCGPCSERRKHLFVQDPQTCK
PDGF-B TVAA-ARPVTRS-PGGSQEQRAKTPQTRVTIRTVRVRRPPKGKHRKFKHTHDKTA
PDGF-A TTSL-NPDYREE-DTGRPRESGKKRKRKRLKPT

                    .         180
hVPF   CSCKNTDSRCKARQLELNERTCRCDKPRR
PDGF-B LKETLGA
```

Fig. 4. Comparison of hVPF and PDGF sequences. The eight conserved cysteines as well as other identical residues of PDGF (*7*) and hVPF are boxed. The potential N-glycosylation sites are denoted by asterisks. Spaces have been provided to yield maximal alignment of the sequences.

sequence of the first five amino acids of bovine VEGF is the same as the sequence of hVPF. The cDNA sequence of human VEGF is similar to that of hVPF (*33*), except for an additional 24 amino acids in the hVPF sequence. The human VPF sequence data will appear in the EMBL/GenBank/DDBJ nucleotide sequence databases under the accession numbers X15997 or M27281.

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31. A Miles-type permeability assay (*19*) was used to detect permeability-enhancing activity. Hairless guinea pigs (IAF/HA-HO; Charles River) were anesthetized by inhalation of methoxyflurane (Metofane; Pitman-Moore). A 1-ml volume of 0.5% (w/v) Evan's blue dye (Sigma) prepared in phosphate-buffered saline (PBS) was injected intracardially into the circulation. Samples for hVPF determination were prepared at appropriate dilutions in PBS, and 200-μl was injected intradermally into sites on the back of the guinea pig. Permeability-enhancing activity was indicated by an intense blue spot at the site of the injection where dye (bound to serum protein) had leaked from the circulation into the tissues.
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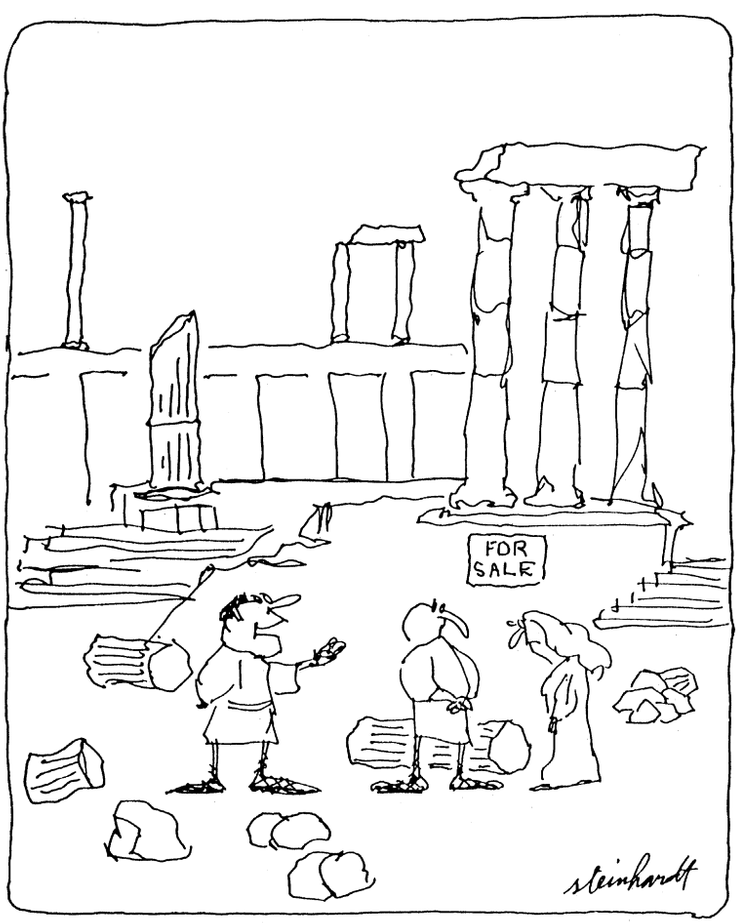

" Sure it looks bad now, but in 2000 years people will come from all over the world to take pictures of this rubble."