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Vascular Permeability Factor (Vascular Endothelial Growth Factor) Gene is Expressed Differentially in Normal Tissues, Macrophages, and Tumors

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Vascular permeability factor (VPF), also known as vascular endothelial growth factor (VEGF), increases microvascular permeability and is a specific mitogen for endothelial cells. Expression of VPF/VEGF previously was demonstrated in a variety of tumor cells, in cultures of pituitary-derived cells, and in corpus luteum. Here we present evidence, by Northern analysis and in situ hybridization, that the VPF/VEGF gene is expressed in many adult organs, including lung, kidney, adrenal gland, heart, liver, and stomach mucosa, as well as in elicited peritoneal macrophages. The highest levels of VPF/VEGF transcripts were found in epithelial cells of lung alveoli, renal glomeruli and adrenal cortex, and in cardiac myocytes. The prominence of VPF/VEGF mRNA in these tissues suggests a possible role for VPF/VEGF in regulating baseline microvascular permeability, which is essential for tissue nutrition and waste removal. We also demonstrate particularly high VPF/VEGF mRNA levels in several human tumors, where it may be involved in promoting tumor angiogenesis and stroma generation, both as an endothelial cell mitogen and indirectly by its permeability enhancing effect that leads to the deposition of a provisional fibrin gel matrix.

INTRODUCTION

Vascular permeability factor (VPF)[1] is a potent mediator of vessel permeability first described in the conditioned media of several rodent and human tumor cell lines (Dvorak *et al.*, 1979; Senger *et al.*, 1983, 1986). VPF is readily detectable in tumor ascites fluids but not in normal serum or plasma. Taking advantage of its heparin-binding properties, VPF has been purified to homogeneity from the serum-free medium of cultured guinea pig tumor cells (Senger *et al.*, 1987, 1990) and human U937 cells (Connolly *et al.*, 1989). Proteins from both sources have a molecular mass of 34–42 kDa and consist of two disulfide-linked subunits with identical N-termini. More recently, a structurally similar protein, identified as a heparin-binding mitogen specific for endothelial cells, was purified from the culture medium of bovine pituitary-derived folliculo-stellate cells (vascular endothelial growth factor or VEGF, Ferrara and Henzel, 1989; folliculo-stellate-derived growth factor or FSdGF, Gospodarowicz *et al.*, 1989) and from rat glioma cells (glioma-derived vascular endothelial growth factor or GD-VEGF, Conn *et al.*, 1990b). Protein sequencing and molecular cloning of human, bovine, and rat cDNAs (Keck *et al.*, 1989; Leung *et al.*, 1989; Tischer *et al.*, 1989; Conn *et al.*, 1990a) demonstrated that tumor VPF, pituitary VEGF/FSdGF, and glioma GD-VEGF are products of the same gene. The sequences also revealed limited but distinct homology of VPF/VEGF to platelet-derived growth factor (PDGF). Alternative forms of the coding regions were isolated from both human and bovine cDNA libraries. Comparison of these cDNA sequences with recently isolated human genomic clones revealed that alternative splicing is involved in VPF/VEGF mRNA biosynthesis (Tischer *et al.*, 1991). Col-

[1] Abbreviations used: bp, base pair; DMEM, Dulbecco's modified Eagle's medium; FSdGF, Folliculo-stellate-derived growth factor; GD-VEGF, glioma-derived VEGF; gVPF, guinea pig VPF; hVPF, human VPF; HBSS, Hank's balanced salt solution; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate; VEGF, vascular endothelial growth factor; VPF, vascular permeability factor.

lectively, the data indicate that at least three polypeptides are encoded by alternatively spliced VPF/VEGF transcript (Figure 1); whether they have different biological activities or distinct subcellular localizations remains to be determined. The splicing pattern is conserved between VPF/VEGF and PDGF (Betsholtz *et al.*, 1990; Tischer *et al.*, 1991).

VPF/VEGF has several biological activities and therefore may serve a variety of functions in vivo (for review, see Senger and Dvorak, 1992). On a molar basis it is ~50 000 times more potent than histamine at increasing microvascular permeability (Senger *et al.*, 1990). In its capacity as both permeability and growth factor, VPF/VEGF very likely is responsible for fluid accumulation in solid and ascites tumors and for initiating and promoting tumor angiogenesis and stroma generation (Nagy *et al.*, 1988). In vitro and at subpicomolar concentrations, VPF/VEGF rapidly increases free cytosolic Ca^{2+} in endothelial cells, apparently by activating a phosphoinositide-specific phospholipase C (Brock *et al.*, 1991). It has been shown also to stimulate von Willebrand factor release from endothelial cells (Brock *et al.*, 1991), to induce expression of endothelial cell tissue factor activity, and to promote monocyte migration (Clauss *et al.*, 1990). Thus, VPF/VEGF may have important roles in processes as diverse as neoplastic growth, wound healing, and inflammation.

To date, expression of VPF/VEGF has been demonstrated in rodent and human tumor cell lines, in bovine and mouse pituitary-derived cells (Ferrara and Henzel, 1989; Gospodarowicz *et al.*, 1989; Plouet *et al.*, 1989), in rat corpus luteum (Phillips *et al.*, 1990), and recently, in cultured vascular smooth muscle cells (Tischer *et al.*, 1991). The goal of the present investigation was to screen VPF/VEGF transcript distribution in a variety of normal adult tissues, cell lines, and autochthonous human tumors by Northern blotting and in situ hybridization. Our results indicate that the VPF/VEGF gene is expressed in many organs in a cell-specific manner. We also demonstrate high VPF/VEGF mRNA levels in several human tumors.

MATERIALS AND METHODS

Animals and Cell Lines

Inbred strain 2 guinea pigs were obtained from the National Cancer Institute (Bethesda, MD). Smooth muscle cells and fibroblasts were explanted from guinea pig aorta and dermis, respectively, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Guinea pig line 10 hepatocarcinoma cells (Dvorak *et al.*, 1979) were cultured in DMEM containing 5% calf serum. Guinea pig resident macrophages were harvested from the peritoneal cavity and lungs by flushing with Hanks' balanced salt solution (HBSS). Alternatively, elicited macrophages were isolated from the peritoneal cavity 4–5 d after intraperitoneal injection of 20 ml sterile mineral oil (Hammond and Dvorak, 1972). Human fibroblasts from colon (CCD18 Co), fibrosarcoma cells (HT1080), nontumorigenic osteosarcoma cells (HOS), and tumorigenic osteosarcoma cells (MNNG HOS) were all obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM containing 10% fetal calf serum.

Polymerase Chain Reaction (PCR) Amplification and DNA Probes

All recombinant DNA manipulations were performed according to Sambrook *et al.* (1989). A guinea pig line 10 cDNA library, prepared from poly(A)$^+$ RNA using λgt11 as a vector, served as a source of gVPF DNA for PCR amplification. PCR reactions were performed in 100 μl using a DNA thermal cycler and AmpliTaq kit (Perkin Elmer-Cetus, Norwalk, CT). Amplification was usually carried out for 30–35 cycles (1 min at 94°C, 2 min at 55°C, 1–3 min at 72°C, depending on the fragment length) followed by 10 min at 72°C. To amplify a fragment from a cDNA phage library, 2×10^6 phage particles were heated at 100°C for 10 min to uncoat and denature phage DNA for use as a template. To amplify a cDNA fragment directly from mRNA, 0.1 μg of total RNA was annealed with random hexanucleotides and reverse transcribed for 30 min at 42°C in a final volume of 20 μl using Mo-MuLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The reaction mixture was then heated at 95°C for 10 min, PCR primers and Taq polymerase were added, the reaction volume was brought up to 100 μl, and PCR amplification was conducted as described above.

We employed the following oligonucleotide primers, which were based on the human VPF cDNA sequence (Keck *et al.*, 1989; Leung *et al.*, 1989):

#1 (forward): 5′-CGCGGATCCAGGAGTACCCTGATGAG-3′

#2 (reverse): 5′-CCGGAATTCACATTTGTTGTGCTGT-3′

The primers have built-in restriction sites (*Bam*HI in primer #1, *Eco*RI in primer #2) at their 5′ ends to facilitate further subcloning. The following VPF-specific probes were prepared by PCR with these primers for use in Northern blot analysis and in situ hybridization: 1) guinea pig VPF (gVPF) probe: the 204-base pair (bp) guinea pig cDNA fragment derived from a guinea pig line 10 cDNA library by amplification with primers #1 and #2 and 2) human VPF (hVPF) probe: the 204-bp human cDNA fragment derived from cell line HT1080 RNA by reverse transcription/PCR with primers #1 and #2.

The PCR products were cloned between the *Eco*RI and *Bam*HI sites of the polylinker region of plasmid pGEM3Zf(+) (Promega, Madison, WI). The cloned inserts were subjected to restriction mapping and double-stranded sequencing by the chain termination method using the Sequenase kit (United States Biochemical, Cleveland, OH). The DNA sequence of the human insert is identical to the previously reported hVPF/VEGF sequence (Keck *et al.*, 1989; Leung *et al.*, 1989). The guinea pig PCR product exhibits very high sequence homology (>90%) with human, as well as bovine and rat, VPF/VEGF cDNAs (Leung *et al.*, 1989; Tischer *et al.*, 1989; Conn *et al.*, 1990a). A clone that encodes complete gVPF polypeptide was isolated later from the same cDNA library (sequence submitted to GenBank database under the accession number M84230). The sequence of the 204-bp guinea pig probe is identical to that contained in the gVPF cDNA clone.

Isolation of RNA From Cells and Tissues and Northern Hybridization

Total RNA was prepared from tissues and cultured cells using the guanidinium isothiocyanate lysis/CsCl gradient method (Sambrook *et al.*, 1989). Animals were killed with ether, and the desired organs were removed, rinsed in phosphate-buffered saline, and immediately frozen in liquid nitrogen. To deplete lungs partially of macrophages, lungs were flushed twice through the trachea with HBSS in situ and then removed. Frozen ovaries pooled from 15 animals were purchased from PelFreez Company (Rogers, AR). For each guinea pig tissue, RNA preparations from at least two animals were analyzed in separate experiments. Human tumors and normal tissues were obtained at surgery and represented portions not required for histopathological diagnosis. Before lysis, tissues were ground in a mortar in the presence of liquid nitrogen. Cells from tissue culture and peritoneal macrophages were washed twice with HBSS and then lysed. After lysis and a CsCl gradient step, the RNA pellet was dissolved in 0.3 M sodium acetate,

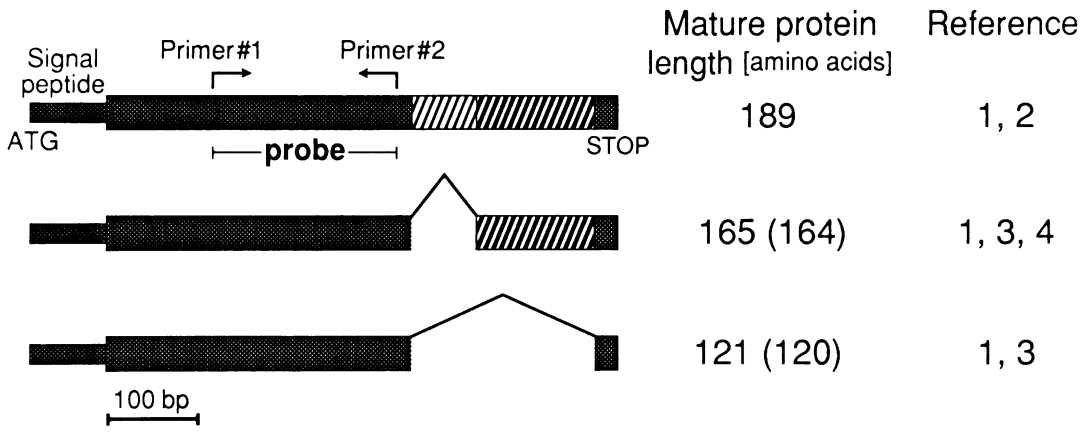

Figure 1. Structure of VPF/VEGF cDNAs, indicating the position of the molecular probes employed in this study. Schematic diagram of VPF/VEGF coding regions of three cDNA variants, corresponding to three alternatively spliced transcripts, as reported by Leung *et al.*, 1989 (1), Keck *et al.*, 1989 (2), Tischer *et al.*, 1989 (3), and Conn *et al.*, 1990a (4). Grey boxes represent sequences common to all variants and hatched boxes represent sequences missing in the two shorter variants. The lengths of polypeptides predicted from the open reading frames in three human cDNA variants are indicated; the numbers in parentheses correspond to open reading frames identified in nonhuman cDNAs, which are one amino acid shorter (lacking 1 residue in position 6 of the mature protein). The positions of PCR primers #1 and #2 used to make probes are marked by arrows.

precipitated with ethanol, and stored as an ethanol precipitate at −80°C.

For Northern analysis, RNA samples that had been normalized for ribosomal RNA content (5–50 μg) were size-fractionated on 1.2% agarose containing 6% formaldehyde and transferred to Nytran membranes (Schleicher and Schuell, Keene, NH). Hybridization was carried out for 24 h at 42°C in 50% formamide, 5× SSPE, 2.5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 10% dextran sulphate (Sambrook *et al.*, 1989). DNA probes were labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, MA) to a specific activity of 1–2 × 10^9 cpm/μg DNA using the random hexamer labeling method (Feinberg and Vogelstein, 1983). Typically, 2–4 × 10^7 cpm of [^{32}P]-labeled probe was used for a 70-cm^2 filter in 10 ml of solution. Although human and guinea pig VPF sequences are >90% homologous and cross-hybridize, species-specific VPF probes were always used to maximize the signal. Final washes were in 0.1× SSC, 0.1% SDS at 65°C. The blots were exposed to Kodak (Rochester, NY) XAR-2 film with an intensifying screen at −70°C for 2–4 d. To control for total mRNA content and lack of degradation in the analyzed preparations, the blots were stripped and subsequently hybridized with a human β-actin probe (2 kb human β-actin cDNA, Clontech Laboratories, Palo Alto, CA).

In Situ Hybridization

Tissues were cut into 1 to 2-mm thick slices and fixed in 85% ethanol, 10% formalin, 5% acetic acid for 3 h, dehydrated in graded alcohols and xylene, and embedded in paraffin. Sections were cut at 5–7 μm, mounted on 3-amino-propyltriethoxy-silane- (Sigma, St. Louis, MO) treated glass slides and rehydrated as described previously (ffrench-Constant *et al.*, 1989). For cytospins, ~10^5 cells were washed with HBSS, centrifuged onto poly-D-lysine-treated slides, submerged in the same fixative for 10 min, and stored in 70% ethanol.

To prepare RNA probes, the pGEM construct with gVPF insert was linearized with *Hin*dIII and transcribed in vitro from the T7 polymerase promoter to yield the antisense probe; alternatively, the same construct was linearized with *Eco*RI and transcribed from the SP6 promoter to yield the sense (control) probe. Transcription reactions were carried out using a Riboprobe Gemini II kit (Promega) in the presence of [α-^{35}S]UTP (Amersham, Arlington Heights, IL). After the template DNA was digested with RQ-1 DNase (Promega), probes were electrophoresed on a urea/acrylamide gel, eluted, and ethanol-precipitated. In situ hybridization was carried out as described previously (ffrench-Constant *et al.*, 1989), except that in some experiments proteinase K concentration was increased 10-fold. RNA probe (typically 3–6 ng at 1 × 10^8 cpm/μg in 50 μl hybridization mixture) was applied to each slide. After hybridization and autoradiography, slides were stained in hematoxylin with or without an eosin counterstain.

RESULTS

Distribution of VPF/VEGF mRNA in Normal Guinea Pig and Human Tissues Detected by Northern Analysis and by In Situ Hybridization

Northern analysis was performed on the RNAs extracted from a number of different normal guinea pig and human tissues. Guinea pig line 10 tumor cells, a rich source of VPF, served as a positive control. The blots were hybridized with VPF-specific probes obtained by PCR amplification of human and guinea pig cDNAs using primers based on human VPF sequence. Identity of the probes was confirmed by DNA sequencing (see MATERIALS AND METHODS). The probes are positioned within a portion of the VPF coding region that is present in all known splicing variants (Figure 1). The probes hybridized discretely to a ~4.5-kb band in line 10 tumor cells and in several normal tissues (Figure 2). When the hybridization signal was particularly strong, a second faint band of ~2.0 kb was also detectable on longer exposures and may represent a degradation product of the larger transcript.

Among the normal guinea pig tissues studied, lung gave the strongest signal (Figure 2A). Other organs that also expressed substantial amounts of VPF mRNA included kidney, adrenal gland, and heart. Lower levels of VPF transcript were found in liver and spleen. We

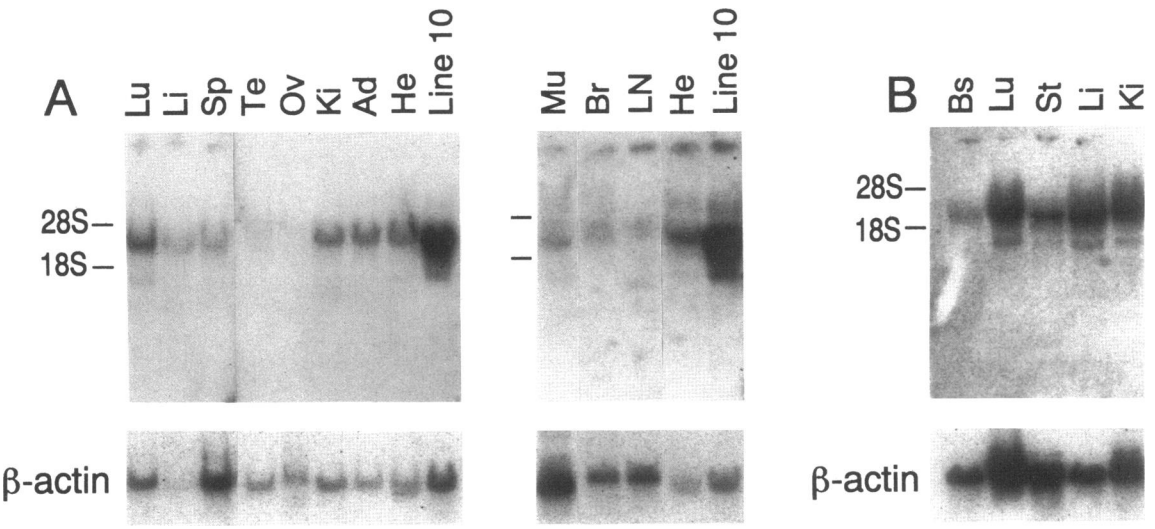

Figure 2. Tissue distribution of VPF mRNA. Northern blots of total RNA sequentially hybridized with VPF (upper) and β-actin (lower) probes. (A) Guinea pig tissues. Left: Lu, lung; Li, liver; Sp, spleen; Te, testis; Ov, ovary; Ki, kidney; Ad, adrenal; He, heart; line 10, hepatocarcinoma cell line (positive control). Right: Mu, skeletal muscle; Br, brain; LN, lymph node; He and line 10, as in left panel. Twenty micrograms of total RNA was loaded in each lane. By ethidium bromide staining of the gel (not shown) the amounts of muscle and liver RNA appeared very similar to those from other tissues. The variation in β-actin signal reflects the proportionally higher expression of this protein in muscle and lower expression in liver. (B) Human tissues: Bs, breast; Lu, lung; St, gastric mucosa; Li, liver; Ki, kidney. Ten micrograms of total breast RNA were loaded; other lanes contain 20 μg total RNA each.

did not obtain convincing evidence for the presence of VPF transcripts in RNA isolated from whole normal guinea pig brain, pituitary, ovary, testis, skin, lymph nodes, stomach, or skeletal muscle. Among normal human tissues examined, lung and kidney contained levels of VPF mRNA similar to those found in the same tissues in guinea pigs. VPF transcript was also readily detected in human liver, gastric mucosa, and, at a lower level, in breast tissue.

Several lines of evidence indicate that the 4.5-kb RNA band identified in the several tissues mentioned above represents the VPF transcript and not that of another gene. First, hybridization reactions were carried out under very stringent conditions (see MATERIALS AND METHODS and Meinkoth and Wahl, 1984). Second, the estimated molecular mass of the detected RNA is similar in guinea pig and human tissues and is very close to the reported size of the hVPF transcript (Leung *et al.*, 1989; Tischer *et al.*, 1991). Finally, this RNA species is particularly abundant in line 10 tumor cells that previously have been shown to secrete large quantities of VPF (Senger *et al.*, 1983). It should be noted that the differences in size among the three splicing variants of this mRNA are too small to be detected by Northern blotting.

To identify cell types responsible for VPF transcription, we applied in situ hybridization with antisense and sense gVPF riboprobes to four guinea pig tissues that were strongly positive by Northern analysis. In normal lung, strong labeling with the antisense probe was observed over cells associated with the luminal surface of alveolar walls, i.e., in the distribution of pulmonary alveolar wall epithelial cells (Figure 3, A and B). In the kidney, label was confined to a population of cells at the periphery of glomerular loops, a location suggestive of glomerular epithelial cells (Figure 3, C–E). In the heart, cardiac myocytes were labeled (Figure 3F). Finally, in sections of adrenal gland, label concentrated over epithelial cells of the outer cortex (Figure 3G). In all instances, grain density with the sense probe was at background levels (e.g., Figure 3H).

Expression of VPF/VEGF mRNA by Freshly Isolated and Cultured Cells

We also evaluated expression of VPF transcripts by Northern analysis of various isolated guinea pig and human cells. These included freshly isolated guinea pig peritoneal macrophages, cultures of guinea pig dermal fibroblasts and aortic smooth muscle cells, human fibroblast cell line CCD 18, and several tumor cell lines. Oil-elicited guinea pig peritoneal macrophages and the tumor cell lines expressed levels of VPF mRNA comparable with those found in lung or kidney and much higher than those in cultures of normal fibroblasts or smooth muscle cells (Figure 4). To demonstrate the presence of VPF mRNA in guinea pig fibroblasts and smooth muscle cells, 50 μg of total RNA in each lane and longer autoradiographic exposure times were required (Figure 4A, right). Culture of elicited guinea pig peritoneal macrophages with phorbol 12-myristate 13-acetate for intervals of 4–24 h did not change their level of VPF expression.

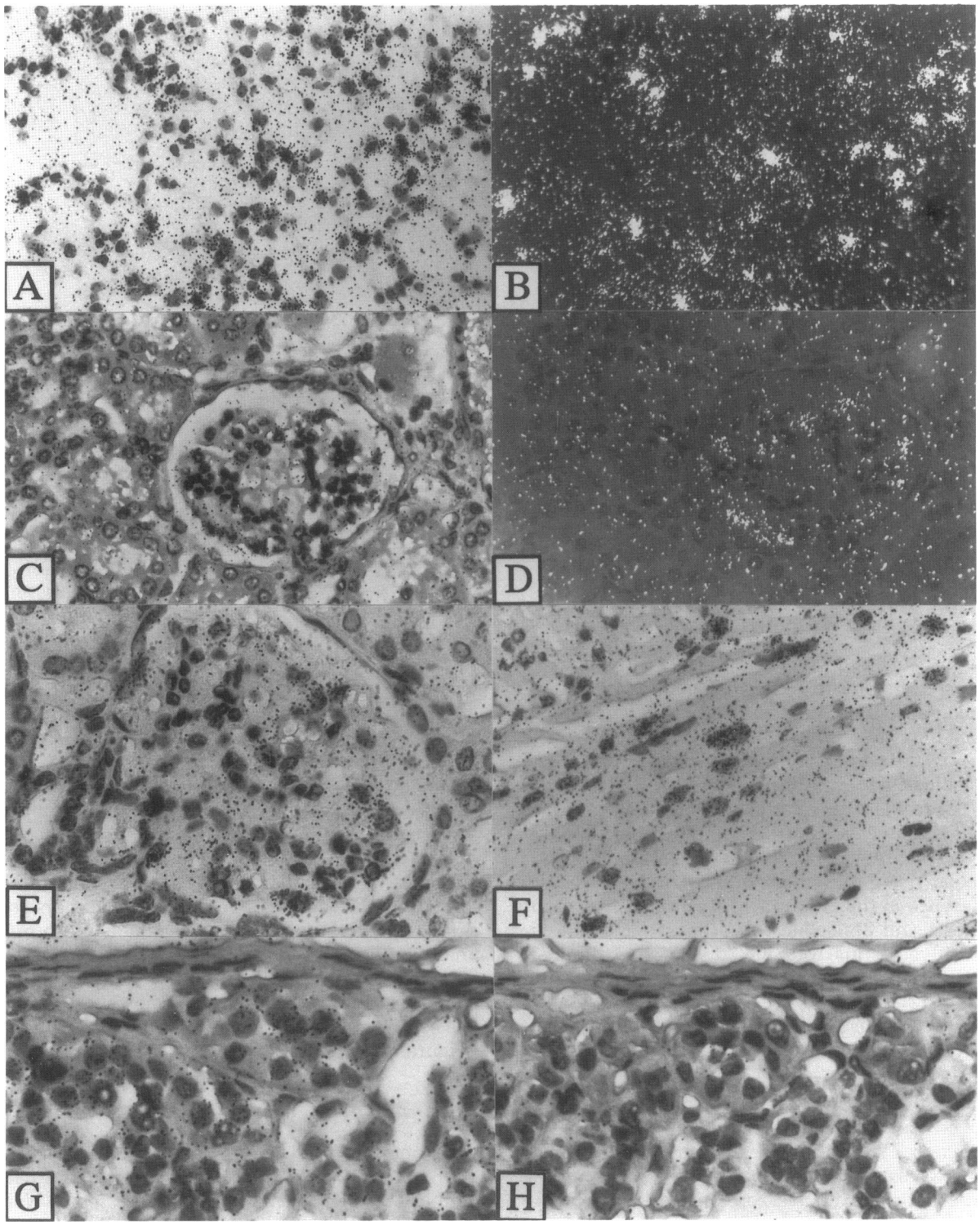

Figure 3. Cellular localization of VPF mRNA expression in guinea pig tissues. In situ hybridization was performed on several normal guinea pig tissues. (A and B) Bright-field and dark-field views of the same lung section hybridized with gVPF antisense probe. Note strong labeling of a population of cells located on the luminal surface of alveoli. Grains are partially obscured by darkly staining nuclei in bright-field photograph (A), but are better appreciated in dark-field photograph (B). (C–E) Bright-field and dark-field views of a section of renal cortex hybridized with gVPF antisense probe. Note labeling of a population of cells within a glomerulus (center of field, C). Label is best seen in corresponding dark-field photograph (D), or in a higher power bright-field view of another glomerulus (E). The labeled cells tend to be located at the periphery of the glomerular tufts. (F) Bright-field view of myocardium. Cardiac muscle cells are labelled. (G and H) Peripheral cortex of adrenal gland. Cortical epithelial cells are labeled with the antisense probe (G), but not with the sense probe (H). Magnifications: A–D, ×430; E, ×680; F–H, ×690.

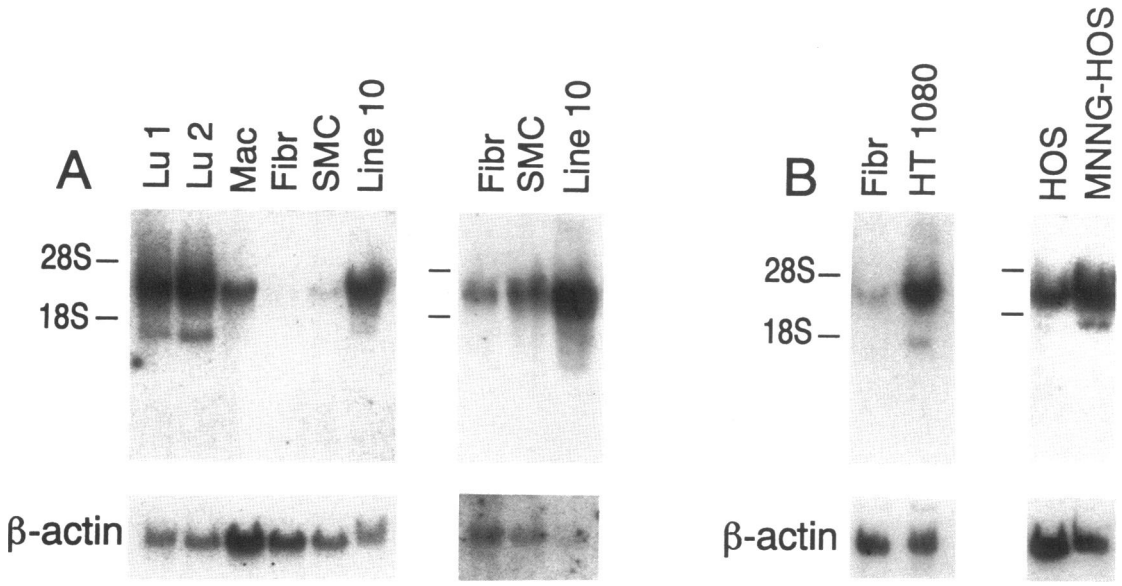

Figure 4. VPF mRNA in macrophages, macrophage-depleted lungs, and isolated cells in culture. Northern blots of total RNA hybridized with VPF and β-actin probes as in Figure 2. (A) Left: guinea pig RNAs. Blots were prepared with 20 μg of RNA from lungs depleted of alveolar macrophages by lavage (Lu1) and from control lungs (Lu2), oil-elicited peritoneal macrophages (Mac), cultured dermal fibroblasts (Fibr), and aortic smooth muscle cells (SMC), and line 10 cells (positive control). Right: the first two lanes each contained 50 μg of RNA compared with 5 μg of line 10 RNA. Autoradiographic exposure was extended to 2 wk to detect low levels of VPF transcript in fibroblasts and smooth muscle cells. (B) RNA from corresponding pairs of human cell lines (20 μg in each lane): normal fibroblast line CCD18 (Fibr) and tumorigenic fibrosarcoma HT1080; nontumorigenic osteosarcoma HOS and a chemically transformed tumorigenic derivative, MNNG-HOS.

We used in situ hybridization to compare VPF expression in resident lung and peritoneal macrophages with that in oil-elicited peritoneal macrophages. By use of the gVPF antisense probe, we observed strong specific labeling of elicited macrophages and line 10 tumor cells but not of resident alveolar or peritoneal macrophages (Figure 5). Moreover, Northern analysis performed on the RNA from lungs extracted before or after depletion of alveolar macrophages by bronchial lavage showed no difference in the amounts of VPF transcript (Figure 4A, lanes 1 and 2). Collectively, the data indicate that resident alveolar macrophages do not contribute significantly to VPF expression in normal lung.

VPF/VEGF Expression in Tumors and Tumor Cell Lines

VPF is secreted by a variety of rodent and human tumor cell lines. By contrast, non-tumorigenic cells in culture have been found to secrete little or no detectable VPF (Senger *et al.*, 1986). To address further the association of VPF expression with neoplastic transformation, we performed Northern analysis on RNA isolated from two matched pairs of nontumorigenic and tumorigenic human cell lines. The VPF mRNA level was substantially elevated in the tumorigenic cell lines (HT 1080 fibrosarcoma, MNNG HOS osteosarcoma) as compared with their nontumorigenic counterparts (Figure 4B).

In preliminary studies that compared human autochthonous carcinomas removed at surgery with corresponding normal tissues derived from the same patient, two of four colonic adenocarcinomas showed clearly increased levels of VPF mRNA compared with adjacent histologically normal bowel mucosa (Figure 6A, cases 3 and 4). One of these two (case 4) also showed increased VPF expression in a metastasis to a lymph node found at the time of surgery. The single gastric adenocarcinoma we studied showed a relatively small increase in VPF mRNA above the level present in adjacent normal gastric mucosa. Of three renal tumors studied, VPF mRNA was increased markedly in one (a predominantly clear cell type, renal cell carcinoma), and it was reduced compared with normal kidney in another (a papillary variant of renal cell carcinoma); VPF transcript was not detectable in a Wilm's tumor of the kidney (Figure 6B).

DISCUSSION

An important finding of the present study is that the VPF gene is expressed in a wide variety of normal adult guinea pig and human tissues. As assessed by Northern analysis, the highest levels of VPF mRNA were found in normal lung, kidney, heart, and adrenal gland. Lower, but still readily detectable, VPF transcript levels were found in liver, spleen, and gastric mucosa, and to a lesser extent in breast. Several other tissues were negative, but Northern analysis may be insufficiently sensitive to detect transcripts produced by only a small

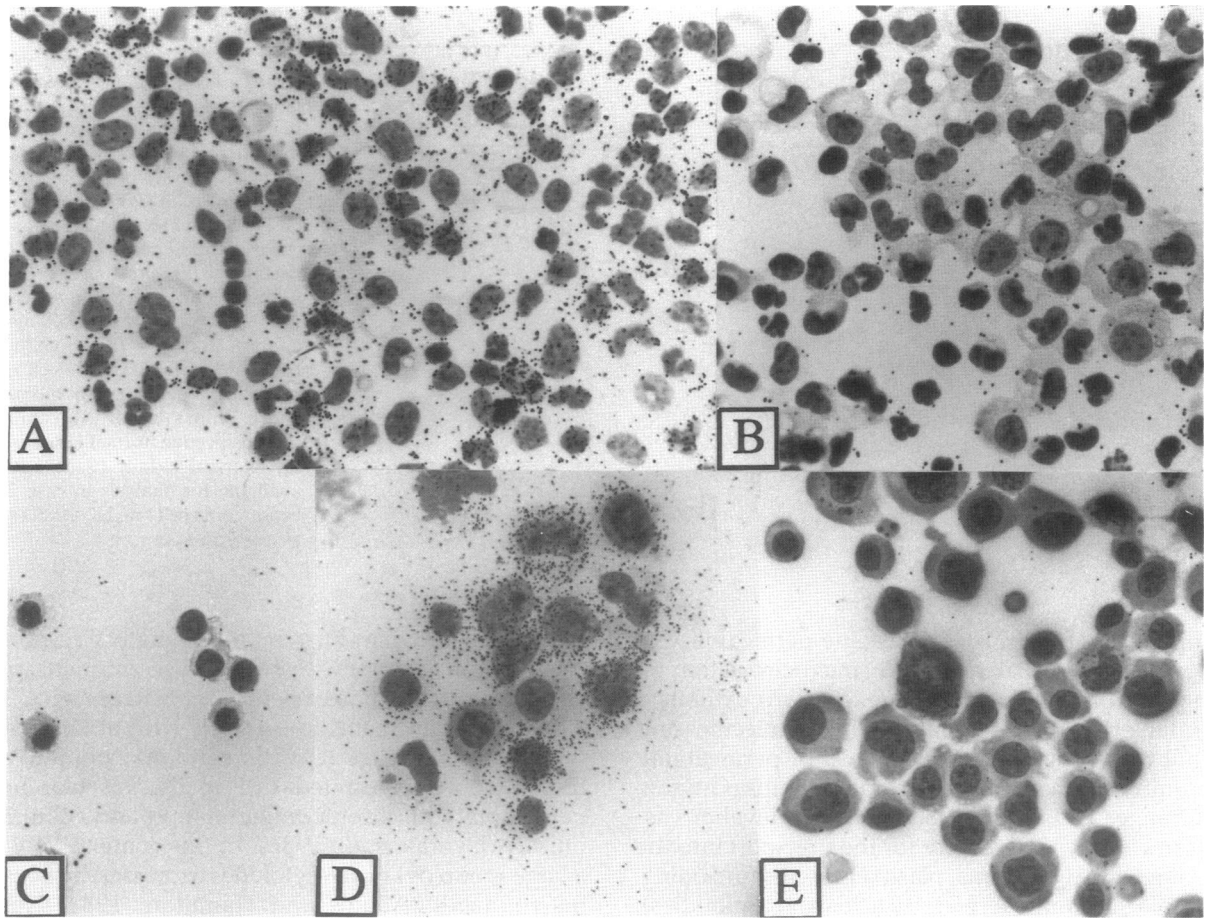

Figure 5. Comparison of VPF expression by elicited and resident guinea pig macrophages and line 10 tumor cells. (A and B). Cytocentrifuge preparations of oil-induced guinea pig peritoneal cells (>80% macrophages) hybridized with gVPF antisense (A) and sense (B) riboprobes. Positive signal is visible with the antisense probe, whereas the sense probe produced only a background signal. Neither resident alveolar macrophages (C) nor resident peritoneal macrophages labeled with the gVPF antisense probe. (D and E) Cytocentrifuge preparations of line 10 tumor cells hybridized with the antisense (D) and sense (E) probe. Note strong labeling with the former but not the latter.

fraction of cells in a heterologous mixture even if those few cells produce significant levels of transcript. Earlier in situ hybridization experiments (Phillips *et al.*, 1990) described VPF transcription in the corpus luteum but not elsewhere in the ovary, suggesting hormonally regulated cell-specific control of VPF expression. Our failure to demonstrate VPF transcripts in RNA prepared from randomly collected guinea pig ovaries very likely reflects the absence of sufficient numbers of corpora lutea in those ovaries for detection by Northern analysis. We have, however, demonstrated the presence of VPF protein in guinea pig corpus luteum by immunohistochemistry (Sioussat, unpublished data), providing support for and supplementing the findings of Phillips *et al.* (1990).

In situ hybridization was employed to define more specifically the source of VPF expression in several of the tissues found positive by Northern blotting. These experiments identified strongly hybridizing subpopulations of cells in the alveolar walls of the lung and in the renal glomeruli; in both instances, the microscopic location of these cells suggests that they are epithelial cells. Outer cortex epithelium of the adrenal gland and cardiac myocytes were also positive for VPF mRNA.

The finding of VPF mRNA in such a diversity of normal tissues provides important insights as to its role in normal physiology. The protein has several known biological activities, the two best characterized being the abilities to increase vascular permeability and to promote endothelial cell proliferation. Phillips *et al.* (1990) have suggested that the VPF synthesized in the ovary by cells of the corpus luteum might be responsible for inducing the substantial angiogenesis characteristic of this structure. However, angiogenesis is not a feature of normal adult lung, kidney, heart, or adrenal gland, and therefore the presence of readily detectable VPF transcripts in these organs requires another explanation. One attractive possibility is that VPF is responsible for

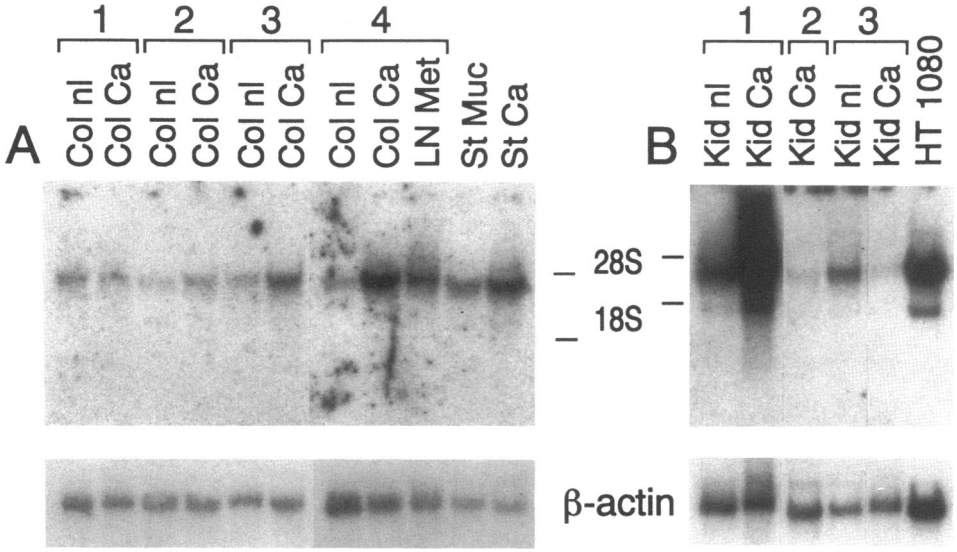

Figure 6. VPF mRNA in human tumors and corresponding normal tissues. Total RNA (20 μg in each lane) from tumors and adjacent normal tissues, hybridized with VPF and β-actin probes. (A) Comparison of four cases (1–4) of colonic adenocarcinoma (Col Ca) and one case of gastric adenocarcinoma (St Ca) with corresponding normal colon (Col nl) or stomach (St Muc) mucosa from the same patient. A lymph node metastasis (LN Met) was available for analysis in case 4. (B) Three kidney tumors (Kid Ca), a clear cell carcinoma (1), a papillary carcinoma (2), and a Wilm's tumor (3), compared with respective normal kidneys (Kid nl). Normal kidney tissue was not available for analysis in case 2. Fibrosarcoma cell line HT 1080 served as a positive control.

inducing and maintaining the baseline permeability of the normal microcirculation. The microcirculation of most normal organs (brain is an exception) exhibits a substantial level of permeability to small molecules and a low but nonetheless significant level of permeability to macromolecules, including plasma proteins (Guyton, 1981; Granger and Perry, 1983). This low level of vascular permeability is necessary for the transport into the tissues of essential nutrients, plasma proteins (including, for example, albumin-borne fatty acids and antibodies), and for clearance of waste products. The kidney is a special case in that the renal glomeruli provide an exceptionally permeable filtration system and many important diseases of the kidney result from impaired glomerular permeability (Cotran *et al.*, 1989). It is therefore of particular interest that VPF expression in the kidney is comparatively high and specifically localized in glomerular epithelium where it is strategically positioned to regulate glomerular filtration. Consistent with relatively high level of VPF transcript in lung and heart, the capillaries of these two organs are reported to be more permeable than those of many other tissues (Guyton, 1981; Granger and Perry, 1983). Our observation that VPF mRNA is synthesized, though at a relatively low level, by cultured smooth muscle cells agrees with the findings of Tischer *et al.* (1991), who have postulated that VPF made by vascular smooth muscle cells plays a role in maintaining the vascular endothelium and in repair of endothelial damage. Thus, VPF may serve both to regulate microvascular permeability and to maintain the existing density of endothelial cells in normal adult tissues. The possibility remains that VPF promotes new blood vessel growth in processes such as embryonic development, neoplasia, and wound healing.

We also detected VPF mRNA in oil-elicited peritoneal macrophages but not in resident peritoneal or alveolar macrophages. Oil induces an inflammatory response in the peritoneal cavity with resulting recruitment, proliferation, and activation of peritoneal macrophages. Thus, VPF expression seems to correlate with at least certain types of macrophage activation and may point to a role for macrophage-secreted VPF in the vascular hyperpermeability of chronic inflammation and cellular immunity (Dvorak *et al.*, 1986). In this context, VPF joins a large group of known cytokines expressed by activated macrophages (Adams and Hamilton, 1984; Nathan, 1987), including fibroblast growth factors, transforming growth factors, and PDGF. It has become increasingly clear that these cytokines have important functions as regulators in both inflammation and tissue repair after wounding.

Three classes of VPF clones that have been isolated from various cDNA libraries (Keck *et al.*, 1989; Leung *et al.*, 1989; Tischer *et al.*, 1989; Conn *et al.*, 1990a; Berse, unpublished data) contain three coding region variants. Tischer *et al.* (1991) have shown that the human VPF/VEGF gene consists of eight exons and that the three VPF/VEGF mRNAs arise by alternative splicing. Theoretically predicted physical properties of the three polypeptides encoded by these mRNA variants differ significantly, but there is no experimental evidence that they correspond to different biological activities. For this study, hybridization probes were designed specifically to detect all VPF mRNAs and did not discriminate among alternatively spliced transcripts. Additional experiments are required to determine whether different cells or tissues express different splicing variants of VPF.

Studies from our laboratory (Nagy *et al.*, 1988; Dvorak *et al.*, 1991) support the hypothesis that VPF plays an important role in aiding growth of some tumors by promoting both tumor angiogenesis and stroma generation. In accord with earlier findings (Senger *et al.*, 1986), VPF

expression was correlated with tumorigenicity in matched pairs of cultured cell lines. As yet preliminary studies of autochthonous tumors surgically removed from cancer patients gave mixed results. In some tumors, VPF expression was increased substantially relative to their normal tissue counterparts, whereas in others, VPF mRNA levels fell below those of corresponding control tissues or were not detected. The high expression of VPF by the clear cell variant of renal cell carcinoma correlates with its prominent vascularity, a typical feature of this tumor (Cotran *et al.*, 1989). In general, analysis of VPF mRNA levels in tumors by Northern blotting requires cautious interpretation. Solid tumors are comprised of both parenchyma (tumor cells) and stroma; if VPF is synthesized only by tumor cells, then Northern analyses performed on whole tumors that also include significant amounts of stroma will inevitably understate the amounts of VPF mRNA actually present in tumor cells. Alternatively, it is possible that only a small fraction of malignant cells in any given tumor synthesizes VPF; again in this case, even if such few cells made abundant VPF mRNA, it might be diluted out and difficult to detect by Northern analysis. In situ hybridization and immunohistochemistry will be helpful in determining with greater precision which tumor cells actually synthesize VPF. For all of these reasons, a much larger and more comprehensive survey of autochthonous tumors will be necessary before meaningful conclusions can be drawn as to the incidence of VPF expression in human malignancy.

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