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Research Article

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Expression of the Vascular Permeability Factor/Vascular Endothelial Growth Factor Gene in Central Nervous System Neoplasms

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Abstract

Expression of the vascular permeability factor/vascular endothelial growth factor (VEGPF) gene was investigated in human central nervous system (CNS) neoplasms and normal brain. Adsorption of capillary permeability activity from human glioblastoma multiforme (GBM) cell conditioned medium and GBM cyst fluids by anti-VEGPF antibodies demonstrated that VEGPF is secreted by GBM cells and is present in sufficient quantities in vivo to induce vascular permeability. Cloning and sequencing of polymerase chain reaction-amplified GBM and normal brain cDNA demonstrated three forms of the VEGPF coding region (567, 495, and 363 nucleotides), corresponding to mature polypeptides of 189, 165, and 121 amino acids, respectively. VEGPF mRNA levels in CNS tumors vs. normal brain were investigated by the RNase protection assay. Significant elevation of VEGPF gene expression was observed in 81% (22/27) of the highly vascular and edema-associated CNS neoplasms (6/8 GBM, 8/8 capillary hemangioblastomas, 6/7 meningiomas, and 2/4 cerebral metastases). In contrast, only 13% (2/15) of those CNS tumors that are not commonly associated with significant neovascularity or cerebral edema (2/10 pituitary adenomas and 0/5 nonastrocytic gliomas) had significantly increased levels of VEGPF mRNA. The relative abundance of the forms of VEGPF mRNA was consistent in tumor and normal brain: $VEGPF_{495} > VEGPF_{363} > VEGPF_{567}$. In situ hybridization confirmed the presence of VEGPF mRNA in tumor cells and its increased abundance in capillary hemangioblastomas. Our results suggest a significant role for VEGPF in the development of CNS tumor neovascularity and peritumoral edema. (*J. Clin. Invest.* 1993. 91:153–159.) Key words: angiogenesis • brain • cerebral edema • glioblastoma • hemangioblastoma

Introduction

Vascular permeability factor/vascular endothelial growth factor (VEGPF)[1] is a secreted 34–43 kD dimeric glycoprotein that induces endothelial cell proliferation, angiogenesis, and capillary permeability. VEGPF has been identified from the conditioned medium of several cell lines and may play a role in the development and maintenance of normal and tumor-associated vasculature (1–13). Cloning studies from several sources demonstrate that this factor exhibits extensive interspecies homology, as well as some homology to platelet-derived growth factor (PDGF) (14–18). Alternative splicing of the human VEGPF gene transcript produces at least three mRNA forms that code for mature polypeptides of 189, 165, and 121 amino acids (11). The physiologic significance of these multiple forms and the patterns of their expression in vivo are not known. VEGPF is distinct among other identified growth factors (epidermal growth factor, acidic fibroblast growth factor [aFGF], basic fibroblast growth factor [bFGF], IL-1, transforming growth factor beta [TGF-β], and PDGF) in that its mitogenic activity is specific for endothelial cells and that it is the only growth factor that exhibits vascular permeability-inducing activity (3). Although this factor has been suggested to be a mediator of tumor-associated angiogenesis and capillary hyperpermeability (3, 19–21), extensive studies of VEGPF gene expression in tumor tissues have yet to be performed.

Much of the morbidity and mortality of malignant, and certain benign central nervous system (CNS) neoplasms is related to the degree of tumor vascularity and the extent of peritumoral vasogenic cerebral edema. Therefore, the unique combination of angiogenic and vascular permeability activities within the same protein, VEGPF, is of particular interest to the study of human CNS neoplasms. Tumor vessels, which arise from angiogenesis, comprise a substantial component of some CNS neoplasms. These vessels are often fragile and exhibit a tendency toward intratumoral hemorrhages. In addition, tumor vessels often lack the tight junctions that are normally present in cerebral microvessels and that constitute a major barrier to the development of vasogenic edema (22–24). The release of vasoactive substance(s) by the tumor cells may also contribute to the development of vasogenic cerebral edema by direct actions on the tumor-associated endothelium (25, 26). A human glioblastoma multiforme (GBM)-derived vascular permeability factor with characteristics similar to VEGPF has been previously described by this laboratory and proposed as a mediator of brain tumor–associated hyperpermeability (26, 27). The possibility that this GBM-derived permeability factor is VEGPF, and the potential association of VEGPF with the development of CNS tumor vascularity and vasogenic edema, prompted this investigation. The goals of this study were (*a*) to determine, using anti-VEGPF antibodies, if GBM-derived permeability factor is VEGPF; (*b*) to determine if there is a correlation in CNS neoplasms between the level of VEGPF mRNA and the degree of tumor-associated vascularity and cerebral edema; and (*c*) to analyze by RNase protection assay (RPA)

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1. *Abbreviations used in this paper:* aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; GBM, glioblastoma multiforme; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; RPA, RNase protection assay; TGF, transforming growth factor; VEGPF, vascular endothelial growth and permeability factor.

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the relative abundance of the different forms of VEGPF mRNA in CNS tumors and normal brain. Because we are studying the multiple forms of VEGPF cDNA and mRNA directly, we have chosen to refer to VEGPF mRNA forms according to the number of nucleotides (567, 495, and 363) in the coding sequences rather than to the corresponding number of amino acids (189, 165, and 121, respectively).

Methods

VEGPF antibodies. A peptide corresponding to the first 20 amino acids of the NH_2 terminus of mature human VEGPF (14, 15) was synthesized by FMOC chemistry with a cysteine at position 21. The peptide was coupled to a carrier protein, keyhole limpet hemocyanin, through the cysteine residue (8). Rabbits were inoculated with the above conjugate and serum was collected. Adsorption of VEGPF was performed as described (8) except for substitution of protein A agarose (Pierce Chemical Co., Rockford, IL) for Staphylococcus A.

Vascular permeability assay. GBM cyst fluids were obtained at surgery and stored at −20°C. Serum-free conditioned medium was concentrated from cultures of GBM cells (U251 MG [28] and SNB lines 78 and 101 [29]) as described (26, 27). The ability of anti-VEGPF antibodies to remove permeability activity from conditioned medium and cyst fluids was assessed by the modified Miles permeability assay (30).

Tissue specimens. 42 human CNS neoplasms and five normal (epileptic) human brain specimens were removed at surgery. Meticulous efforts were made to isolate the tumor tissue from surrounding normal or gliotic brain. Specimens were quick frozen and immediately stored at −70°C. The histopathological diagnoses were established using standard criteria (31). In addition, two samples of total RNA isolated from normal human adult brains were purchased from Clonetech Laboratories (Palo Alto, CA).

Identification of VEGPF cDNA. Total RNA was isolated from GBM and normal brain (32). First strand cDNA was synthesized with superscript RNase H reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) using a cDNA synthesis kit and according to the manufacturer's instructions (Pharmacia, Piscataway, NJ). Oligonucleotide primers with EcoRI or HindIII restriction sites were synthesized identical to the 5′ end (5′-ATATAGAATTCGCACCCATGGCAGAAGGAGG-3′) or complementary to the 3′ end (5′-ATATAAAGCTTCCGCCTCGGCTTGTCACATC-3′) of the coding region of mature human $VEGPF_{567}$ (14, 15). Polymerase chain reactions (PCR) were performed with the following thermocycle parameters: 94°C for 1 min, 53°C for 2 min, 72°C for 3 min for 45 cycles with a 5-s auto extension. PCR products were digested with EcoRI and HindIII and cloned into pBluescript II SK+ (Stratagene Inc., La Jolla, CA) (33). Insert-positive plasmids were sequenced on double-stranded templates by the dideoxynucleotide chain-termination method using Sequenase kit (United States Biochemical Corp., Cleveland, OH).

RPA. Radiolabeled riboprobes were synthesized with [α^{32}P]CTP from linearized plasmid DNA using the in vitro RNA transcription method (33). Riboprobes were labeled to a specific activity of $\sim 5 \times 10^8$ dpm/μg. Control unlabeled sense RNA was synthesized from linearized DNA templates as described above. RPA was performed according to the manufacturer's instructions (RPA II; Ambion, Inc., Austin, TX). Samples of total RNA (10 μg) from 42 human CNS neoplasms and seven normal brain samples were hybridized to completion (> 8 h) with molar excesses of riboprobes VEGPF (10^5 cpm) and β-actin ($2.0–2.5 \times 10^5$ cpm). Both radiolabeled riboprobes were added to each RNA sample to eliminate inaccuracies in RNA loading. Single-stranded RNA was removed by digestion with RNase T1 (1/50) for 45 min at 37°C followed by ethanol precipitation in the manufacturer's buffer with the addition of 20 μg transfer RNA/sample (RNase A was omitted because it results in digestion of the control sense VEGPF mRNA:riboprobe hybrids at a "breathing" site of nine consecutive AT pairs beginning with nucleotide 341 of $VEGPF_{567}$). The samples were denatured and protected species separated on a 6% polyacrylamide sequencing gel in 1× TBE (100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.3) for 2 h at 50 A. The gels were exposed to Kodak XAR film with intensifying screens for 2 h (β-actin) and 16 h (VEGPF) at −70°C.

Densitometric analysis. Densitometry was performed on a video densitometer (model 620; Bio-Rad Laboratories, Richmond, CA) and analyzed with data analysis software (1D Analyst II; Bio-Rad Laboratories). The optical density was determined for a 2-h exposure of the β-actin band at 210 bases and for a 16-h exposure of the VEGPF band at 344 bases. U251 RNA (10 μg) was included with each experiment to serve as an internal control.

In situ hybridization. Tissue sections from two capillary hemangioblastomas, one somatotroph pituitary adenoma, and one normal brain were used for in situ hybridization to DNA oligonucleotide probes either complementary or identical to 41 bases between nucleotides 242–282 of $VEGPF_{567}$. This sequence is present in all three forms of VEGPF. Oligonucleotides were labeled at the 3′ end to a tail length of 25–30 bases with α(thio)^{35}S-dATP using deoxynucleotide terminal transferase (New England Nuclear, Boston, MA). Tissue preparation and hybridization were performed as described (34).

Southern hybridization. Matched tumor and lymphocyte DNAs (10 μg) were digested with TaqI in the manufacturer's recommended buffer (Bethesda Research Laboratories), fractionated on a 0.8% agarose gel, and transferred to Nytran. Cloned $VEGPF_{567}$ plasmid insert was radiolabeled by random priming to a specific activity of $\sim 10^9$ dpm/μg and hybridized to the membrane for 24 h. Membranes were washed under stringent conditions and exposed to Kodak XAR film for appropriate intervals. All procedures were performed according to standard techniques (33).

Results

Identification of VEGPF in conditioned medium and cyst fluids. Previous results from this laboratory demonstrated that human GBM-derived vascular permeability activity shares many characteristics with VEGPF identified from other sources (26, 27). To determine directly if the GBM-derived permeability activity is attributable to VEGPF, we raised antibodies to the 20 NH_2-terminal amino acids of mature human VEGPF and examined the ability of these antibodies to remove the permeability-inducing activity from GBM cell conditioned medium and cyst fluids. Anti-VEGPF antibodies removed 75–99% of the permeability activity present in these sources (Table I). The residual permeability activity may be caused by the inability of this antibody to recognize all conformations of VEGPF, or to the presence of other minor permeability factors in the conditioned medium and cyst fluids.

PCR amplification and cloning of VEGPF. Oligonucleotide primers corresponding to the 5′ and 3′ ends of the published sequence for $VEGPF_{567}$ cDNA were used in a PCR reaction with template cDNA synthesized from GBM and normal brain RNA. A major band (495 bp) and two minor bands (567 and 363 bp) were present in both sources. The PCR products were amplified, cloned, and sequenced, and found to be identical to those sequences already reported for human VEGPF (14, 15). In addition, a novel 267-bp sequence from a GBM tumor was also cloned. It contains a 300-bp deletion spanning nucleotides 185–484. This form maintains the reading frame but substitutes threonine for asparagine at codon 62.

VEGPF mRNA levels in CNS neoplasms and normal brain. Because Northern blot analysis does not distinguish between the multiple forms of the VEGPF coding region, we used the RPA technique to examine the different forms of VEGPF

Table I. Adsorption of Human GBM-Derived Permeability Activity by Anti-VEGPF Antibodies

Sample	IgG	Permeability activity*
Conditioned medium	None	220±43
U251		
U251	Preimmune	208±34
U251	Anti-VEGPF	24±22
U251	Anti-VEGPF (+ VEGPF peptide)	213±26
SNB 78	Preimmune	232±27
SNB 78	Anti-VEGPF	28±24
SNB 101	Preimmune	305±35
SNB 101	Anti-VEGPF	54±28
Cyst fluids	Preimmune	178
GMB #1		
GBM #1	Anti-VEGPF	47
GBM #2	Preimmune	170
GBM #2	Anti-VEGPF	2

* Permeability activity is expressed as the $A_{620} \times 10^3$. GBM cell conditioned medium and cyst fluids were incubated with immobilized IgG from either preimmune serum or anti-VEGPF antiserum and assayed in the Miles capillary permeability assay. Data are expressed as the mean±SD ($n \geq 3$) for conditioned media or as the average of duplicate determinations for cyst fluids. Preincubation of anti-VEGPF IgG with the synthetic NH_2-terminal peptide of VEGPF was performed to confirm the specificity of the anti-VEGPF antibody.

mRNA directly. As a positive control for the RPA, unlabeled sense strand RNA synthesized from the cloned DNA templates of $VEGPF_{567}$, $VEGPF_{495}$, $VEGPF_{363}$, and $VEGPF_{267}$ was hybridized to radiolabeled $VEGPF_{567}$ riboprobe (Fig. 1 *A*). The size of the riboprobe fragments protected by the four VEGPF mRNA forms agreed with the predicted results. Hybridization of $VEGPF_{567}$ RNA protected 567 bases of the riboprobe. Hybridization of both $VEGPF_{495}$ RNA and $VEGPF_{363}$ RNA protected a riboprobe fragment of 344 bases. This common band results from the identical 5′ ends and shared splice site of $VEGPF_{495}$ and $VEGPF_{363}$. A band of 151 bases results from protection of the riboprobe by the 3′ portion of $VEGPF_{495}$ RNA. $VEGPF_{267}$ RNA protected a fragment of 184 bases at the 5′ end and of 83 bases at the 3′ end of the riboprobe.

The levels of the different forms of VEGPF mRNA were determined in 42 CNS tumors and seven normal brain samples. The tumors were categorized according to whether they are commonly associated with a high (group A) or low (group B) degree of neovascularity and vasogenic cerebral edema. There were 27 group A tumors: eight GBM, eight capillary hemangioblastomas, seven meningiomas, and four cerebral metastases (two adenocarcinomas and two melanomas); and 15 group B tumors: 10 pituitary adenomas (four Cushing's and one Nelson's corticotroph adenomas, three somatotroph adenomas; one thyrotroph adenoma, and one nonsecreting adenoma) and five nonastrocytic gliomas (three oligodendrogliomas, one ependymoma, and one radiation-induced gliosarcoma). Hybridization of total RNA to the $VEGPF_{567}$ riboprobe generated three bands of 567, 344, and 151 bases in

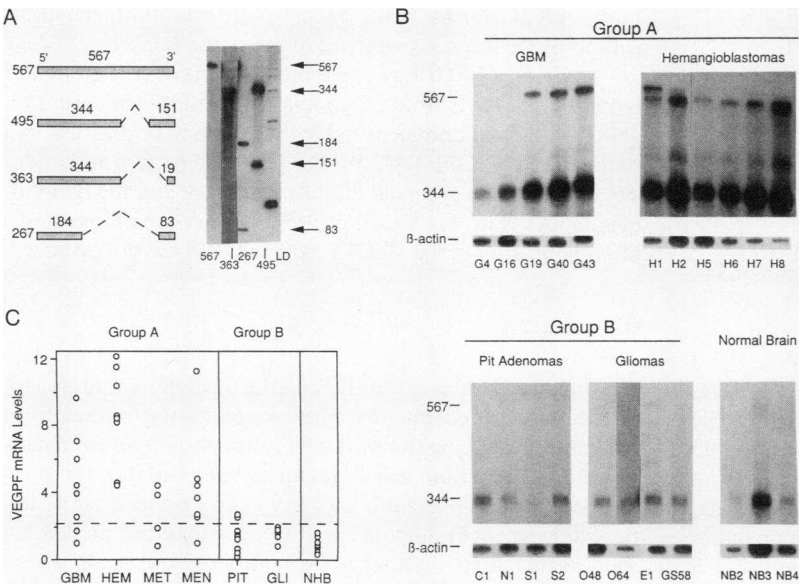

Figure 1. Expression of VEGPF mRNA in CNS tumors and normal brain. (*A*, *left*) The four forms of the VEGPF coding region (the number of nucleotides is indicated on the left) that were cloned from human GBM cDNA are shown schematically to indicate the positions of the internal deletions (*dashed lines*). The numbers over the shaded bars indicate the size of the riboprobe fragments which should be protected by hybridization of each form to a radiolabeled $VEGPF_{567}$ riboprobe. (*A*, *right*) Control sense strand RNA was synthesized from each of the four VEGPF clones, hybridized to radiolabeled $VEGPF_{567}$ riboprobe, treated with RNase and analyzed on a 6% polyacrylamide gel. The size of the unlabeled sense strand is indicated under the appropriate gel lane, and the resulting protected riboprobe fragments are identified by the arrows. A labeled 123-bp DNA ladder (*LD*) is also shown (RNA runs ~ 5–10% slower than DNA). (*B*) Total RNA (10 μg) from CNS neoplasms and normal brain samples were hybridized to radiolabeled $VEGPF_{567}$ and mouse β-actin riboprobes and processed as described in Methods. The tumors are grouped by their common association with either a high (group A) or low (group B) degree of tumor neovascularity and vasogenic edema. The size of the protected riboprobe fragments is indicated on the left (the band of 151 bases is not shown). The β-actin bands at 210 bases are also shown. An additional band of ~ 610 bases present in a few samples results from protection of the full length riboprobe by undigested DNA template. Pituitary adenomas: *C1*, Cushing's corticotroph adenoma; *N1*, Nelson's corticotroph adenoma; and *S1* and *S2*, somatotroph adenomas. Nonastrocytic gliomas: *O48* and *O64*, oligodendrogliomas; *E1*, ependymoma; and *GS58*, gliosarcoma. (*C*) The optical densities of the 344-base VEGPF band and 210-base β-actin band were obtained for the 49 tissue specimens as described in Methods. Each sample was analyzed in one to four experiments, and the mean VEGPF OD/β-actin OD for each sample obtained (expressed as *VEGPF mRNA Levels*). *HEM*, hemangioblastomas; *MET*, cerebral metastases; *MEN*, meningiomas; *PIT*, pituitary adenomas; *GLI*, nonastrocytic gliomas; and *NHB*, normal human brain. Significant elevation of VEGPF gene expression is defined as levels of VEGPF mRNA that exceed the levels in normal brain by > 2.5 SD (*horizontal dashed line*).

all of the samples. The band of 344 bases, resulting from protection of the riboprobe by both $VEGPF_{495}$ and $VEGPF_{363}$ mRNA, was the predominant species in all of the tissues examined. When compared to the level of VEGPF mRNA in normal brain, higher levels of VEGPF mRNA were observed in the group A tumors. Representative samples from each tumor group and from normal brain are shown in Fig. 1 *B*. Bands corresponding to hybridization of $VEGPF_{267}$ mRNA to the $VEGPF_{567}$ riboprobe were not observed in any of the samples.

VEGPF mRNA was quantified by comparing the optical density of the major band of 344 bases to the optical density of a β-actin band in each sample. Hybridization of human RNA to the radiolabeled mouse β-actin riboprobe protected multiple size fragments between 200–240 bases. The relative abundance of these different actin bands was consistent throughout the samples, so a band of 210 bases was selected for densitometry. The ratios of VEGPF/β-actin mRNA for the 42 CNS neoplasms and the seven normal brain samples are shown in Fig. 1 *C*. Of all the tumor types, capillary hemangioblastomas had the highest level of VEGPF mRNA, averaging ninefold higher than normal brain (8.5±2.9 vs. 0.9±0.5, mean±SD; $P < 0.001$). Significantly higher levels of VEGPF mRNA were also observed in 6/8 GBM, 6/7 meningiomas, and 2/4 cerebral metastases (we define significant elevation as a level of VEGPF mRNA that exceeds the average level in normal brain by > 2.5 SD). Overall, 81% (22/27) of the group A tumors had elevated levels of VEGPF gene expression, averaging sixfold higher (5.4±3.5; $P = 0.002$) than normal brain. In contrast, only 13% (2/15) of the group B tumors (2/10 pituitary adenomas and 0/5 nonastrocytic gliomas) had significant elevation in the level of VEGPF mRNA, with the average group B level (1.3±0.8) not significantly different from normal brain ($P = 0.23$).

Identification of $VEGPF_{363}$ mRNA by RPA. Hybridization of total RNA with the radiolabeled $VEGPF_{567}$ riboprobe does not distinguish between the individual contributions of $VEGPF_{495}$ and $VEGPF_{363}$ mRNA to the band of 344 bases (Fig. 1 *A*). Therefore, radiolabeled riboprobe synthesized from the cloned $VEGPF_{495}$ DNA template was hybridized to total RNA from 15 tumors (three GBM, three capillary hemangioblastomas, one cerebral metastasis, one meningiomas, four pituitary adenomas, and three nonastrocytic gliomas) and two normal brain samples. Under these conditions, the band of 344 bases results from protection of the $VEGPF_{495}$ riboprobe by $VEGPF_{363}$ mRNA only. The 344-base band was detected in all 17 RNA samples, indicating that $VEGPF_{567}$, $VEGPF_{495}$, and $VEGPF_{363}$ mRNA forms are present in all of the CNS tissues examined. Representative samples are shown in Fig. 2. The intensity of the 344 band was less than the intensity of the 495 band in 15/17 specimens, allowing us to conclude that $VEGPF_{495}$ is the predominant form of VEGPF in these specimens. In addition, the extremely low intensity of the band of 567 bases (representing $VEGPF_{567}$ mRNA) shown in Fig. 1 *B* suggests that the relative abundance of the forms of VEGPF mRNA in the majority of the samples is: $VEGPF_{495} > VEGPF_{363} > VEGPF_{567}$.

Analysis of VEGPF expression by in situ hybridization. Radiolabeled oligonucleotide probes complementary or identical to a segment of the VEGPF nucleotide sequence common to the three forms of VEGPF mRNA were hybridized to tissue sections from two capillary hemangioblastomas, one pituitary

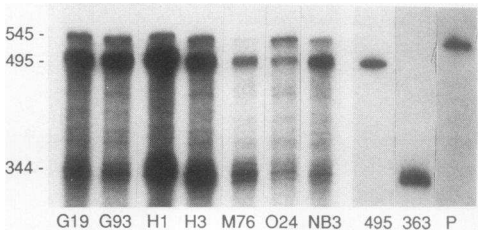

Figure 2. Expression of $VEGPF_{363}$ mRNA. To specifically determine the presence of $VEGPF_{363}$ mRNA, a radiolabeled $VEGPF_{495}$ riboprobe was hybridized to total RNA (10 μg) from CNS tumors and normal brain. The size of the protected riboprobe fragment is indicated on the left. The band of 495 bases results from hybridization of $VEGPF_{567}$ and $VEGPF_{495}$ mRNA to the riboprobe. The band of 344 bases can result only from protection of the riboprobe by $VEGPF_{363}$ mRNA. The band of 545 bases corresponds to protection of the full length riboprobe by undigested DNA template. Samples from two GBM (*G19* and *G93*), two hemangioblastomas (*H1* and *H3*), one metastatic melanoma (*M76*), one oligodendroglioma (*O24*) and one normal brain (*NB3*) are shown. Unlabeled sense strand RNA synthesized from cloned DNA templates of $VEGPF_{495}$ and $VEGPF_{363}$, and undigested radiolabeled $VEGPF_{495}$ riboprobe (*P*) served as positive controls.

macroadenoma, and one normal brain. The tissue sections were counterstained with hematoxylin and eosin. Three of the samples are shown in Fig. 3. Abundant hybridization of the antisense VEGPF probe was evident in the tissue sections from the capillary hemangioblastomas. However, while hybridization to stromal cells was clearly evident, whether the VEGPF mRNA is expressed exclusively by the stromal cells or by both stromal and endothelial cells was not discernible in these sections. Only scant hybridization occurred in the pituitary adenoma and the normal brain tissues. There was minimal hybridization of the sense probe in all of the tissue samples.

Analysis of VEGPF gene organization in GBM. Tumor and lymphocyte DNAs from 22 glioblastoma multiforme were digested with TaqI and analyzed by Southern hybridization to a radiolabeled VEGPF DNA probe. The same pattern and intensity of bands was observed in both the tumor and the lymphocyte DNAs (Fig. 4). This indicates that gross rearrangements or amplification of the VEGPF gene in GBM has not occurred.

Discussion

A correlation between VEGPF and the formation of tumor-induced vasogenic edema was first suggested by the findings from an ascites-producing tumor model in which protein extravasation into ascites fluid was dramatically decreased in the presence of anti-VEGPF antibodies (19). This correlation is supported by recent findings in a non-CNS solid tumor model that showed increased vascular permeability only in those vessels around which VEGPF concentrations were high (21). We have previously reported that human GBM cyst fluids and conditioned medium from cultured primary brain tumors contain vascular permeability-inducing activity, and that this activity shares many characteristics with VEGPF (26, 27). Adsorption of this GBM-derived vascular permeability activity by specific anti-VEGPF antibodies demonstrates definitively that (*a*) human GBM produce VEGPF in vitro and in vivo and (*b*) most

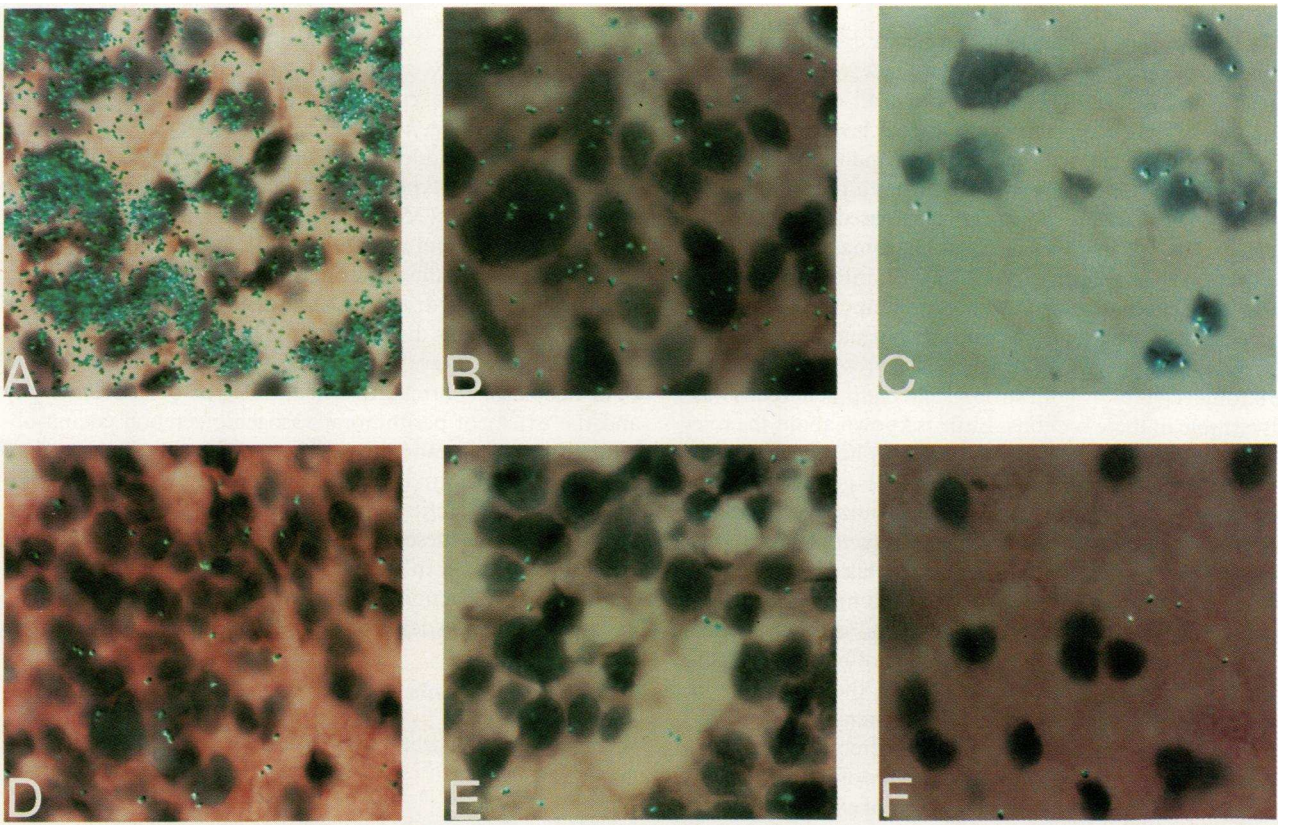

Figure 3. Analysis of VEGPF mRNA expression by in situ hybridization. Radiolabeled oligonucleotide probes complementary (antisense) or identical (sense) to 41 bases of the VEGPF coding region were hybridized to tissue sections, analyzed by emulsion autoradiography, and counterstained with hematoxylin and eosin. Photographs were taken at ×1,000 using polarized light epiluminescence. Hybridization of antisense probe (*A–C*) or control sense probe (*D–F*) to tissue specimens from a capillary hemangioblastoma (*A* and *D*), pituitary adenoma (*B* and *E*), and normal brain (*C* and *F*) are shown.

of the permeability-inducing activity produced by GBM cells is attributable to VEGPF. A role for VEGPF in the development of clinically significant vasogenic cerebral edema is further suggested by our finding of elevated VEGPF expression in GBM, cerebral metastases, and meningiomas, tumors that frequently exhibit clinically significant peritumoral cerebral edema. In contrast, the level of VEGPF gene expression in CNS neoplasms that are not associated with cerebral edema (pituitary adenomas and nonastrocytic gliomas) was similar to the level of expression in normal brain.

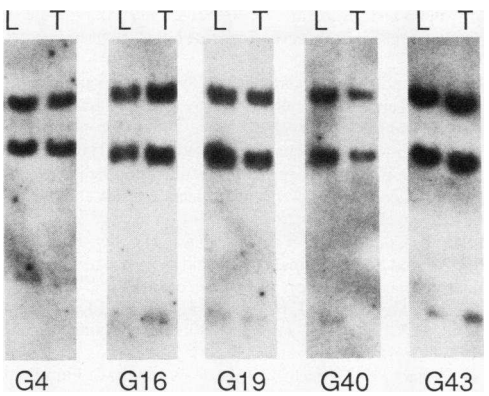

Figure 4. Southern hybridization of VEGPF probe to genomic DNA. Matched lymphocyte and tumor DNAs from glioblastoma multiforme were processed as described in Methods and filters hybridized to a radiolabeled $VEGPF_{567}$ DNA probe. Five GBM samples (corresponding to those in Fig. 1 *B*) are shown. *L*, lymphocyte DNA; *T*, tumor DNA.

VEGPF is mitogenic for endothelial cells at concentrations significantly lower than that required to increase vascular permeability (3, 4). This suggests that the concentration of VEGPF in GBM cysts is likely sufficient to induce endothelial cell proliferation. In fact, in this study, the correlation between VEGPF gene expression and tumor vascularity was stronger than was the correlation between VEGPF expression and cerebral edema. For example, capillary hemangioblastomas, which are one of the most vascular neoplasms in the CNS, exhibited the highest levels of VEGPF mRNA. However, these tumors are rarely associated with clinically significant cerebral edema. In contrast, metastatic tumors, which are not as vascular as capillary hemangioblastomas but are often associated with severe cerebral edema, had much lower levels of VEGPF mRNA compared to the hemangioblastomas. A more complete understanding of the contribution of VEGPF to the development of both tumor-associated edema and neovascularization awaits elucidation of the mechanisms by which this factor acts on both normal cerebral and CNS tumor-associated capillary endothelial cells.

Capillary hemangioblastomas are one of the most common primary infratentorial neoplasms in adults. Grossly, the tumors are cherry red, reflecting their highly vascular composi-

tion. Microscopically, hemangioblastomas are comprised of numerous endothelial-lined capillaries with intervening lipid-laden stromal cells (35, 36). In this study, all eight capillary hemangioblastomas had significantly higher levels of VEGPF mRNA than did the normal brain samples and the 15 group B tumors in which extensive vascularity is not a prominent feature. In situ hybridization confirms the increased abundance of VEGPF mRNA in capillary hemangioblastomas, and demonstrates that the vast majority of the VEGPF mRNA is present within tumor cells and not infiltrating mononuclear cells, reactive astrocytes, normal endothelium, or normal brain parenchyma. Whether the VEGPF mRNA is expressed exclusively by the stromal cells or by both stromal and endothelial cells was not discernible in these sections. Little is known about the role of other angiogenic growth factors in capillary hemangioblastomas. One report examining aFGF and bFGF gene expression in a variety of CNS tumors by in situ hybridization, demonstrated expression of these two angiogenesis factors in all of the tumor types except the capillary hemangioblastoma (37).

Glioblastoma multiforme is the most common and most malignant primary adult CNS neoplasm and is distinguished by its high degree of tumor neovascularity, often forming glomerular vascular tufts (31, 36). As a result, the expression of angiogenesis factors has been investigated extensively in GBM, and to a lesser extent in meningiomas and cerebral metastases, CNS tumors also associated with a high degree of tumor neovascularity. To varying degrees, elevated levels of expression of the genes for PDGF, epidermal growth factor, TGF-α, TGF-β, aFGF, bFGF, and now VEGPF, have been reported in GBM, gliomas, meningiomas, and cerebral metastases (38–45). These results suggest that the expression of multiple angiogenic growth factors supports the development and maintenance of these tumors. However, the elevated level of VEGPF gene expression in GBM does not appear to result from amplification or rearrangement of the VEGPF gene (Fig. 4).

We used RPA to examine directly the expression of the different coding sequences of VEGPF in CNS neoplasms and normal brain. With this technique, we show that at least three forms of VEGPF mRNA are present, and that the relative abundance of each form is maintained in the CNS specimens: $VEGPF_{495} > VEGPF_{363} > VEGPF_{567}$. This suggests that regulation of splicing may be the same in both tumor and normal tissues, and that the expression of all three forms of VEGPF may be functionally important. This suggestion is supported by the finding that VEGPF protein may exist as a heterodimer in vivo, which would require simultaneous expression of the multiple forms of VEGPF (46). We identified by PCR amplification of GBM cDNA a fourth form of VEGPF cDNA ($VEGPF_{267}$) containing a 300-bp internal deletion. Since this form was not detected by RPA, the significance of this novel form remains to be determined. Recently, a $VEGPF_{618}$ clone containing a 51-bp insertion was amplified and sequenced from cDNA of fetal liver (47). In our assay, hybridization of either $VEGPF_{618}$ mRNA or $VEGPF_{567}$ mRNA to the largest radiolabeled riboprobe ($VEGPF_{567}$) would protect a band of 567 bases. Therefore, we would not have differentiated $VEGPF_{618}$ mRNA from $VEGPF_{567}$ mRNA in our assay. However, the consistently low intensity of the 567 band in all of the tissues examined suggests that if $VEGPF_{618}$ is present in these CNS tumors or normal brain, it is expressed at very low levels.

Investigation of VEGPF gene expression in non-CNS tumors has recently been reported. High levels of VEGPF mRNA were observed in 3/5 gastrointestinal adenocarcinomas and 1/3 renal carcinomas (18). Significantly, the highest level of VEGPF gene expression was observed in a clear cell carcinoma of the kidney. This tumor is a highly vascular neoplasm that is phenotypically indistinguishable from a capillary hemangioblastoma (35, 36). In addition, multiple clear cell renal carcinomas and capillary hemangioblastomas occur together in patients suffering from the inherited genetic disorder, von Hippel-Lindau disease. The contribution of VEGPF to the development of this disease needs further investigation.

The increased level of VEGPF mRNA in several human CNS neoplasms correlates with the degree of neovascularity and the extent of peritumoral vasogenic cerebral edema observed in these tumor types. The consistently high level of VEGPF mRNA found in all of the capillary hemangioblastomas may reflect a critical and specific role for VEGPF in the development of these highly vascular tumors. Analysis of VEGPF expression in tumors and elucidation of the mechanisms of VEGPF action may identify select targets for blocking vasogenic cerebral edema or inhibiting tumor neovascularity.

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References

1. Ferrara, N., and W. J. Henzel. 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 161:851–858.

2. Gospodarowicz, D., J. A. Abraham, and J. Schilling. 1989. Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliclulo stellate cells. *Proc. Natl. Acad. Sci. USA.* 86:7311–7315.

3. Connolly, D. T., D. M. Heuvelman, R. Nelson, J. V. Olander, B. L. Eppley, J. J. Delfino, N. R. Siegel, R. M. Leimgruber, and J. Feder. 1989. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J. Clin. Invest.* 84:1470–1478.

4. Connolly, D. T., J. V. Olander, D. Heuvelman, R. Nelson, R. Monsell, N. Siegel, B. L. Haymore, R. Leimgruber, and J. Feder. 1989. Human vascular permeability factor: isolation from U937 cells. *J. Biol. Chem.* 264:20017–20024.

5. Levy, A. P., R. Tamargo, H. Brem, and D. Nathans. 1989. An endothelial cell growth factor from the mouse neuroblastoma cell line NB41. *Growth Factors.* 2:9–19.

6. Plouet, J., J. Schilling, and D. Gospodarowicz. 1989. Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3801–3806.

7. Conn, G., D. D. Soderman, M-T. Schaeffer, M. Wile, V. B. Hatcher, and K. A. Thomas. 1990. Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. *Proc. Natl. Acad. Sci. USA.* 87:1323–1327.

8. Senger, D. R., D. T. Connolly, L. Van De Water, J. Feder, and H. F. Dvorak. 1990. Purification and NH_2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability factor. 1990. *Cancer Res.* 50:1774–1778.

9. Rosenthal, R. A., J. F. Megyesi, W. J. Henzel, N. Ferrara, and J. Folkman. 1990. Conditioned medium from mouse sarcoma 180 cells contains vascular endothelial growth factor. *Growth Factors.* 4:53–59.

10. Clauss, M., M. Gerlach, H. Gerlach, J. Brett, F. Wang, P. C. Familletti, Y.-C. E. Pan, J. V. Olander, D. T. Connolly, and D. Stern. 1990. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *J. Exp. Med.* 172:1535–1545.

11. Tischer, E., R. Mitchell, T. Hartman, M. Silva, D. Gospodarowicz, J. C. Fiddes, and J. A. Abraham. 1991. The human gene for vascular endothelial growth factor. *J. Biol. Chem.* 266:11947–11954.

12. Myoken, Y., Y. Kayada, T. Okamoto, M. Kan, G. H. Sato, and J. D. Sato.

1991. Vascular endothelial cell growth factor (VEGPF) produced by A-431 human epidermoid carcinoma cells and identification of VEGPF membrane binding sites. *Proc. Natl. Acad. Sci. USA.* 88:5819–5823.

13. Ferrara, N., J. Winer, and T. Burton. 1991. Aortic smooth muscle cells express and secrete vascular endothelial growth factor. *Growth Factors.* 5:141–148.

14. Leung, D. W., G. Cachianes, W-J. Kuang, D. V. Goeddel, and N. Ferrara. 1989. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science (Wash. DC).* 246:1306–1309.

15. Keck, P. J., S. D. Hauser, G. Krivi, K. Sanzo, T. Warren, J. Feder, and D. T. Connolly. 1989. Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science (Wash. DC).* 246:1309–1312.

16. Tischer, E., D. Gospodarowicz, R. Mitchell, M. Silva, J. Schilling, K. Lau, T. Crisp, J. C. Fiddes, and J. A. Abraham. 1989. Vascular endothelial growth factor: a new member of the platelet-derived growth factor gene family. *Biochem. Biophys. Res. Commun.* 165:1198–1206.

17. Conn, G., M. L. Bayne, D. D. Soderman, P. W. Kwok, K. A. Sullivan, T. M. Palisi, D. A. Hope, and K. A. Thomas. 1990. Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA.* 87:2628–2632.

18. Berse, B., L. F. Brown, L. Van De Water, H. F. Dvorak, and D. R. Senger. 1992. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Mol. Biol. Cell.* 3:211–220.

19. Senger, D. R., S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. S. Harvey, and H. F. Dvorak. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science (Wash. DC).* 219:983–985.

20. Senger, D. R., C. A. Perruzzi, J. Feder, and H. F. Dvorak. 1986. A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. *Cancer Res.* 46:5629–5632.

21. Dvorak, H. F., T. M. Sioussat, L. F. Brown, B. Berse, J. A. Nagy, A. Sotrel, E. J. Manseau, L. Van De Water, and D. R. Senger. 1991. Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. *J. Exp. Med.* 174:1275–1278.

22. Long, D. M. 1970. Capillary ultrastructure and the blood-brain barrier in human malignant brain tumors. *J. Neurosurg.* 32:127–144.

23. Hirano, A., and T. Matsui. 1975. Vascular structures in brain tumors. *Hum. Pathol.* 6:611–621.

24. Lantos, P. L., P. J. Luthert, and B. R. Deane. 1985. Vascular permeability and cerebral oedema in experimental brain tumours. *In* Brain Edema. Y. Inaba, I. Klatzo, and M. Spatz, editors. Springer-Verlag, Berlin, Germany. 40–47.

25. Baethmann, A. 1978. Pathophysiological and pathochemical aspects of cerebral edema. *Neurosurg. Rev.* 1:85–100.

26. Bruce, J. N., G. R. Criscuolo, M. J. Merrill, R. R. Moquin, J. B. Blacklock, and E. H. Oldfield. 1987. Vascular permeability induced by protein product of malignant brain tumors: inhibition by dexamethasone. *J. Neurosurg.* 67:880–884.

27. Criscuolo, G. R., M. J. Merrill, and E. H. Oldfield. 1988. Further characterization of malignant glioma-derived vascular permeability factor. *J. Neurosurg.* 69:254–262.

28. Westermark, B., J. Ponten, and R. Hugosson. 1973. Determinants for the establishment of permanent tissue culture lines from human gliomas. *Acta Pathol. Microbiol. Scand. Sect. A Pathol.* 81:791–805.

29. Merrill, M. J., and N. A. Edwards. 1990. Insulin-like growth factor-I receptors in human glial tumors. *J. Clin. Endocrinol. & Metab.* 71:199–209.

30. Udaka, K., Y. Takeuchi, and H. Z. Movat. 1970. Simple method for quantitation of enhanced vascular permeability. *Proc. Soc. Exp. Biol. Med.* 133:1384–1387.

31. Burger, P. C., and F. S. Vogel. 1982. Surgical Pathology of the Nervous System and its Coverings. 2nd ed. Wiley, New York, N.Y.

32. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

33. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

34. Young, W. S. III, T. I. Bonner, and M. R. Brann. 1986. Mesencephalic dopamine neurons regulate the expression of neuropeptide mRNAs in the rat forebrain. *Proc. Natl. Acad. Sci. USA.* 83:9827–9831.

35. Rengachary, S. S. 1985. Hemangioblastomas. *In* Neurosurgery. R. H. Wilkins, and S. S. Rengachary, editors. McGraw-Hill Book Co., New York. 772–782.

36. Okazaki, H., and B. W. Scheithauer. 1988. Atlas of Neuropathology. Gower Medical Publishing, New York. 139 pp.

37. Akutsu, Y., T. Aida, S. Nakazawa, and G. Asano. 1991. Localization of acidic and basic fibroblast growth factor mRNA in human brain tumors. *Jpn. J. Cancer Res.* 82:1022–1027.

38. Hermansson, M., M. Nister, C. Betsholtz, C-H. Heldin, B. Westermark, and K. Funa. 1988. Endothelial cell hyperplasia in human glioblastoma: coexpression of mRNA for platelet-derived growth factor (PDGF) B chain and PDGF receptor suggests autocrine growth stimulation. *Proc. Natl. Acad. Sci. USA.* 85:7748–7752.

39. Maxwell, M., S. P. Naber, H. J. Wolfe, T. Galanopoulos, E. T. Hedley-Whyte, P. M. Black, and H. N. Antoniades. 1990. Coexpression of platelet-derived growth factor (PDGF) and PDGF-receptor genes by primary human astrocytomas may contribute to their development and maintenance. *J. Clin. Invest.* 86:131–140.

40. Takahashi, J. A., H. Mori, M. Fukumoto, K. Igarashi, M. Jaye, Y. Oda, H. Kikuchi, and M. Hatanaka. 1990. Gene expression of fibroblast growth factors in human gliomas and meningiomas: demonstration of cellular source of basic fibroblast growth factor mRNA and peptide in tumor tissues. *Proc. Natl. Acad. Sci. USA.* 87:5710–5714.

41. Zagzag, D., D. C. Miller, Y. Sato, D. B. Rifkin, and D. E. Burstein. 1990. Immunohistochemical localization of basic fibroblast growth factor in astrocytomas. *Cancer Res.* 50:7393–7398.

42. Schlegel, U., P. L. Moots, M. K. Rosenblum, H. T. Thaler, and H. M. Furneaux. 1990. Expression of transforming growth factor alpha in human gliomas. *Oncogene.* 5:1839–1842.

43. Maxwell, M., S. P. Naber, H. J. Wolfe, E. T. Hedley-Whyte, T. Galanopoulos, J. Neville-Golden, and H. N. Antoniades. 1991. Expression of angiogenic growth factor genes in primary human astrocytomas may contribute to their growth and progression. *Cancer Res.* 51:1345–1351.

44. Ekstrand, A. J., C. D. James, W. K. Cavenee, B. Seliger, R. F. Pettersson, and V. P. Collins. 1991. Genes for epidermal growth factor receptor, transforming growth factor α, and epidermal growth factor and their expression in human gliomas *in vivo*. *Cancer Res.* 51:2164–2172.

45. Saxena, A., and I. U. Ali. 1992. Increased expression of genes from growth factor signaling pathways in glioblastoma cell lines. *Oncogene.* 7:243–247.

46. Yeo, T-K., D. R. Senger, H. F. Dvorak, L. Freter, and K-T. Yeo. 1991. Glycosylation is essential for efficient secretion but not for permeability-enhancing activity of vascular permeability factor (vascular endothelial growth factor). *Biochem. Biophys. Res. Commun.* 179:1568–1575.

47. Houck, K. A., N. Ferrara, J. Winer, G. Cachianes, B. Li, and D. W. Leung. 1991. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol. Endocrinol.* 5:1806–1814.