Expression of vascular endothelial growth factor-b in human astrocytoma¹

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Growth of human malignant gliomas is stringently dependent on an angiogenic process that probably involves vascular endothelial growth factor (VEGF). Expressions of mRNA coding for the different forms of VEGF were analyzed in surgical specimens from human astrocytomas. Low levels of placental growth factor (PGF) and VEGFC mRNA were observed in polymerase chain reaction, but not in Northern blot experiments. VEGF mRNA was found in some but not all grade and grade IV astrocytomas. VEGFB mRNA was observed in all tissue samples analyzed irrespective of the tumor grade. A new splice variant of VEGFB (VEGFB₁₅₅) that lacks exons 5 and 6 is described. Expressions of VEGF mRNA in cultured glioblastomas cells were upregulated by hypoxia, but the sensitivity of the cells to hypoxia was reduced as compared with normal rat astrocytes. VEGF expression was depressed by dexamethasone. Expressions of VEGFB mRNA were affected neither by hypoxia nor by dexamethasone. The results indicate a coexpression of VEGF mRNA and VEGFB mRNA in human astrocytomas. Expression of VEGFB is markedly different from that of VEGF. Possible roles of VEGFB as a cofactor for

hypoxia-induced angiogenesis in human astrocytomas are discussed. *Neuro-Oncology* 2, 80-86, 2000 (Posted to *Neuro-Oncology [serial online]*, Doc. 99-33, March 7, 2000. URL <neuro-oncology.mc.duke.edu>)

VEGF³ is a dimeric glycoprotein that acts as a selective mitogen for vascular endothelial cells and as a vascular permeability factor (Connolly et al., 1989; Leung et al., 1989). It binds to and induces biological responses via two tyrosine kinase receptors: Flt1 (DeVries et al., 1992) and Flk1/KDR (Terman et al., 1992). Vascular permeability activity and immunoreactive VEGF are found in brain cyst fluids (Berkman et al., 1993; Weindel et al., 1994). VEGF expression is upregulated in high-grade astrocytomas that are highly vascularized and edema associated and is specifically localized in tumor cells that are in the direct proximity of necrotic areas (Phillips et al., 1993; Plate et al., 1992; Shweiki et al., 1992). It has been proposed that local hypoxia adjacent to necrotic areas within tumors triggers VEGF expression and induces vascularization of these areas (Plate et al., 1992; Shweiki et al., 1992). This hypothesis is supported by the observation that hypoxia induces VEGF mRNA and protein expression in cultured tumoral or nontumoral cells (Goldberg and Schneider, 1994; Ladoux and Frelin, 1993; Shweiki et al., 1992).

Recent studies have identified four other members in the VEGF gene family: placental growth factor (PGF) (Maglione et al., 1991, 1993), VEGFB (Olofsson et al., 1996a), VEGFC (Joukov et al., 1996), and VEGFD (Farnebo et al., 1999). The existence of multiple forms of VEGF that heterodimerize with VEGF and that recognize partially overlapping sets of receptors calls for a reevaluation of the role of these factors in human astrocytoma. This study compares the expressions of mRNA for VEGF, PGF, VEGFB, and VEGFC in human brain astrocytomas and analyses the regulation of the expres-

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³Abbreviations used are as follows: GAPDH, glyceraldehyde-3-phosphodeshydrogenase; MEM, modified Eagle's medium; PCR, polymerase chain reaction; PGF, placental growth factor; SDS, sodium dodecyl sulfate; VEGF, vascular endothelial growth factor.

sion of VEGF and VEGFB in cultured human astrocytoma cells.

Materials and Methods

Materials and Specimen Selection

Chemicals were from Sigma Chemical Company (St. Louis, Mo.) unless otherwise indicated.

Brain tumor specimens obtained in patients who underwent surgery at Nice Hospital were retrospectively selected for analysis. Surgical specimens were immediately immersed in Dulbecco's MEM supplemented with 400 units/ml penicillin and 200 µg/ml streptomycin and brought to the laboratory. They were then cut into three pieces under sterile conditions. One piece of tissue was immediately frozen in liquid nitrogen and used for RNA extraction. An adjacent piece was fixed in 15% formalin and used for histologic analysis. The third piece was used to isolate cells. Histologic typing was established according to current principles of neuropathology, and grading was based on the World Health Organization classification of brain tumors. All procedures were performed according to the rules of the Ethical Committee of the National Center for Scientific Research.

Cell Cultures

Cell suspensions were prepared from surgical specimens using a grinder sieve, were washed of contaminating blood and debris, and were seeded into 25-cm² culture flasks using Dulbecco's MEM supplemented with 2 mM glutamine, 10% heat-inactivated fetal bovine serum (Roche Diagnostics, Meylan, France), 10 ng/ml epidermal growth factor, 5 mM sodium pyruvate, 400 units/ml penicillin, and 200 µg/ml streptomycin. Culture media were renewed twice a week. At confluence, cultures were dispersed using trypsin (2 mg/ml) in phosphate-buffered saline supplemented with 200 mg/ml EDTA. Suspended cells were seeded in the same culture medium at a split ratio of 2 and maintained in vitro for up to 40 passages. Rat C6 glioma cells (Chassande et al., 1988), rat brain capillary endothelial cells (Vigne et al., 1989), and cortical astrocytes from newborn rat cortex (Marsault et al., 1990) were prepared as previously described. The culture medium was Dulbecco's MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Hypoxic conditions were generated as previously described (Eyssen-Hernandez et al., 1996). Briefly, the cells were washed with serum-free medium, incubated in Dulbecco's MEM supplemented with 1% fetal bovine serum for 24 h in a humidified atmosphere containing 5% CO₂, further incubated for 6 h in an air-tight container (Institut Gustave Roussy, Villejuif, France), and continuously flushed with a mixture of gases containing 5% CO₂ and 95% N₂.

RNA Isolation and Northern Blots

Tissue samples (10–60 mg) were minced with a sterile scalpel and homogenized with 1 ml TRIzol Reagent (Life

Technologies, Cergy Pontoise, France) in a 2-ml Dounce homogenizer (Polylabo, Strasbourg, France). Extraction was then performed according to the manufacturer's instructions. RNA was extracted from cultured cells according to Chomczynski and Sacchi (1987).

RNA samples (10 µg) were subjected to a denaturing electrophoresis on a 1.2% (wt/vol) agarose gel and transferred to Nytran membranes (Schleicher and Schuell, Cera Labo, Ecqueville, France). Blots were hybridized with VEGF, PGF, and GAPDH probes at 42°C in the presence of 30% formamide, 5× saline-sodium phosphate EDTA, 5× Denhardt's, 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA. Blots were hybridized with VEGFB and VEGFC probes at 47°C in the presence of 50% formamide, 5× saline-sodium phosphate EDTA, 5× Denhardt's, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA. After hybridization, blots were washed twice with 2× saline-sodium phosphate EDTA containing 0.1% SDS at 42°C for 15 min. Autoradiography was performed for 2 days with intensifying screens at –80°C using Kodak X-Omat AR films. Quantification of the hybridization signals was performed using a Phosphorimager (Fuji Medical Systems, Stamford, Conn.). Signals were normalized to that of GAPDH.

The VEGF probe was a 605-bp fragment corresponding to the whole VEGF₁₆₅ human coding sequence. The PGF probe was a 470-bp fragment (nucleotides 321–791, Genbank 354936). The VEGFB probe was a 678-bp fragment (nucleotides 1–678, Genbank U43368). The VEGFC probe was a 1481-bp fragment (nucleotides 352–1833, Genbank 394216). The GAPDH probe was kindly provided by Dr. F. Moreau-Gachelin. The 18S ribosomal RNA probe was a 98-bp fragment corresponding to a conserved region in mammals (Raynal et al., 1984). Probes were labeled with ³²P using the multiprimer DNA labeling system (Amersham, Les Ulis, France). Specific activities were >5 × 10⁸ cpm/µg DNA.

PCR

Reverse transcription was performed using 500 ng total RNA, 10 µg/ml oligo(dT)₁₂₋₁₈, 200 units/assay Moloney leukemia virus reverse transcriptase (Life Technologies), and deoxyribonucleoside triphosphates (0.5 mM each). Reaction was carried out for 1 h at 37°C.

PCR was performed using 1/10 of the reverse transcription volume, 400 µM deoxyribonucleoside triphosphates, primers (4 ng/µl, Genset, Paris, France), and 20 units/ml Goldstar DNA polymerase (Eurogentec, Seraing, Belgium) in a final volume of 25 µl. Primers, annealing temperatures, and product lengths are listed in Table 1. After heating the samples for 1 min at 95°C, 30 cycles of amplification (1 min heating at 95°C, 1 min annealing, and 1.30 min elongation at 72°C) were performed. Samples were then left at 72°C for 10 min. PCR products were subcloned into the insert site of pTag vector (kit LigA-Tor; R&D Systems, Abingdon, U.K.) and sequenced using the *Taq* dye deoxy terminator sequencing system (kit Prism; Perkin Elmer, Les Ulis, France) and an Applied Biosystems (Les Ulis, France) 373A DNA Sequencer.

Table 1. Primers and annealing temperatures used in PCR experiments

	Primers	Annealing temperature	Product size (bp)
VEGF	Sense (5'-CCATGAACCTTCTGCTCTCTTG-3')	52°C	704 (VEGF ₁₈₉)
	Antisense (5'-GGTGAGAGRTCTRGTTCCCGA-3')		632 (VEGF ₁₆₅)
PGF	Sense (5'-GATGCCGGTCATGAGGCTGT-3')	59°C	500 (VEGF ₁₂₁)
	Antisense (5'-CTCTCCTCCAAGGGGTGGGT-3')		470 (PGF ₁₄₉)
VEGFB	Sense (5'-CCATGAGCCCTCTGCTCCGCC-3')	60°C	533 (PGF ₁₇₀)
	Antisense (5'-GCCATGTGTACCTTCGCAGC-3')		678 (VEGFB ₁₈₆)
VEGFC	Sense (5'-ATGCACTTGCTGGGCTTCTTC-3')	57°C	577 (VEGFB ₁₆₇)
	Antisense (5'-TCTTGCTGTTGGTCATTGG-3')		541 (VEGFB ₁₅₅)
			1481

Abbreviations: PCR, polymerase chain reaction; VEGF, vascular endothelial growth factor; PGF, placental growth factor.

Southern Blots

PCR products were loaded onto a 1% agarose gel column containing ethidium bromide and transferred onto Nytran N Plus membranes (Schleicher and Schuell). Hybridization was carried out with the appropriate probe as described in the section on Northern blots.

Results

Expression of VEGF Genes in Brain Tumors

We analyzed 37 brain tissue samples. They comprised 1 normal tissue (hippocampal cortex from cortectomy for temporal lobe epilepsy), 3 meningiomas, 8 grade II astrocytomas, 8 grade III astrocytomas, and 17 glioblastomas. Expression of *VEGF*, *VEGFB*, *VEGFC*, and *PGF* was analyzed using Northern blot experiments. Data obtained are summarized in Table 2.

VEGF mRNA was mainly observed in grade III and IV astrocytomas (Fig. 1). The major forms found were VEGF₁₂₁ and VEGF₁₆₅ (Fig. 2). We observed, however, that transcripts coding for VEGF₁₂₁ and VEGF₁₆₅, which were negative in Northern blot experiments, were detected in grade II astrocytomas. Very low levels of expression were found in the normal brain tissue and in grade I astrocytomas.

Differences in hybridization signals were not due to loading errors as evidenced by 18S ribosomal RNA signals. These results, although nonquantitative, suggest

that VEGF mRNA is expressed in all brain tissues examined and is upregulated in high-grade astrocytomas.

Messenger RNA for VEGFB (1.4 kb) was detected in almost all samples analyzed, including low-grade astrocytomas and one sample of normal brain tissue (Table 2). Fig. 3 shows a representative Northern blot. PCR experiments (Fig. 4) further indicated the presence of two transcripts coding for VEGFB₁₈₆ and VEGFB₁₆₇ in representative tissue samples.

Sequences corresponding to PGF₁₄₉, PGF₁₇₀, and VEGFC were amplified by PCR in all tissue samples analyzed (data not shown). The corresponding mRNA was not detected in Northern blots experiments.

Taken together, these results indicate that all forms of VEGF mRNA can be detected in human astrocytomas by PCR. Only VEGF mRNA and VEGFB mRNA are detected in Northern blot experiments. Expression of VEGFB mRNA is ubiquitous. Expression of VEGF mRNA is markedly increased in grade III and IV astrocytomas.

Identification of a New Splicing Variant of VEGFB

PCR amplification products have been systematically sequenced to look for possible mutations. All sequences corresponded to the published sequences. A novel form of VEGFB was identified, however, in cultured cells isolated from a glioblastoma. The 155 amino acid sequence is shown in Fig. 5. It corresponded to a form of VEGFB

Table 2. Expressions of mRNA for VEGF, VEGFB, VEGFC, and PGF in tumor tissue samples

Tissue	VEGF	VEGFB	VEGFC	PGF
Normal	0/1	1/1	0/1	0/1
I	0/3	3/3	0/3	0/3
II	1/6	6/7	0/7	0/3
III	3/8	6/6	0/6	0/7
IV	10/17	15/16	0/16	0/9

This summary considers the results of only Northern blots and excludes PCR data that were positive for VEGFC and PGF.

Abbreviations: See Table 1.

Fig. 1. Expression of VEGF in human astrocytomas. Representative Northern blots of total RNA were obtained from different brain tissue samples. Grades of the tumor samples are indicated on the top of the lanes. N, normal tissue; VEGF, vascular endothelial growth factor.

Fig. 2. Expression of VEGF splice variants in human astrocytomas. Representative Southern blots show expressions of VEGF₁₂₁ and VEGF₁₆₅. Grades of the tumor samples are indicated on the top of the lanes. See Fig. 1 for abbreviations.

that lacks exons 5 and 6. Splicing introduced a frameshift and the use of a different open reading frame. As a consequence, the C-terminal sequences of VEGFB₁₅₅ and VEGFB₁₈₆ are widely divergent. Splicing of exon 6, which generates VEGFB₁₆₇, induced a similar frameshift. As a consequence, C-terminal sequences of VEGFB₁₆₇ and VEGFB₁₅₅ are identical.

Effects of Hypoxia and Dexamethasone on VEGF and VEGFB Expressions by Cultured Human Astrocytoma Cells

Cells were isolated from surgical specimens corresponding to different grades and grown in vitro. Expressions of VEGF mRNA were analyzed using Northern blot experiments. Expressions of VEGF mRNA appeared more ubiquitous in cultures than in tissue samples. It was not observed in two cell cultures isolated from grade I tumors. It was observed in 1 isolate out of 3 from grade II tumors, in 3 isolates out of 4 grade III tumors, and in 5 isolates out of 8 glioblastomas (data not shown).

Expression of VEGFB mRNA was detected in all cells analyzed. VEGFC mRNA was observed in cultured cells derived from grade III (2/2) and grade IV (1/3) astrocytomas. PGF mRNA was detected only in PCR experiments.

Fig. 6 compares expressions of VEGF mRNA and VEGFB mRNA in normoxic and hypoxic cultures of glioblastoma cells, of rat C6 astrocytoma cells, of normal rat astrocytes, and of rat brain capillary endothelial cells. Hypoxia induced VEGF mRNA expression in all cell types analyzed, but to variable extents. Human glioma cells, C6 glioma cells, and endothelial cells were the least sensitive. Normal rat astrocytes were two times more sensitive to hypoxia. These indicated that the sensitivity of glioma cells to hypoxia is not greater than that of untransformed cells. VEGFB mRNA expression was insensitive to hypoxia (Fig. 6).

Fig. 7 shows that dexamethasone (1 μ M) decreased VEGF mRNA expression by 40% to 70% in glioblastoma cells. It had no action on VEGFB mRNA expression. Thus, dexamethasone selectively reduced VEGF mRNA expression.

Fig. 3. Expression of VEGFB in human astrocytomas. Representative Northern blots of total RNA were obtained from different brain tissue samples. Grades of the tumor samples are indicated on the top of the lanes. See Fig. 1 for abbreviations.

Discussion

Previous studies have documented expression of VEGF (Chan et al., 1998; Machein et al., 1999; Schmidt et al., 1999) and PGF in human primary brain tumors (Nomura et al., 1998). This study confirmed the presence of low amounts of PGF in human astrocytomas by PCR experiments, but not by Northern blot experiments. VEGF mRNA was detected using PCR in all samples analyzed, but a hybridization signal could only be obtained with grade III and IV astrocytomas using Northern blot experiments. Thus, our results confirmed increased VEGF mRNA expression during the anaplastic progression of human astrocytomas.

VEGFB is widely expressed in rat tissues and is most prominent in heart and skeletal muscle (Olofsson et al., 1996a). Expression of VEGFB mRNA has previously been documented in normal mouse brains (Lagercrantz et al., 1998). It was also reported in human tumors of noncerebral origin (Salven et al., 1998). It has not yet been reported in normal human brain tissues. This study documents a prominent expression of VEGFB mRNA in all brain tissue samples analyzed: normal brain tissue, low-grade astrocytomas, and high-grade astrocytomas. Two forms of VEGFB have previously been described: VEGFB₁₈₆, which is encoded by unspliced transcripts, and VEGFB₁₆₇, which lacks exon 6 and differs from VEGFB₁₈₆ in its C-terminal sequence due to a frameshift and a different open reading frame and stop codon (Olofsson et al., 1996b). These two forms are present in the tissue samples analyzed. This work further identifies a

Fig. 4. Expression of VEGFB splice variants in human astrocytomas. Representative Southern blots show expressions of VEGF₁₆₇ and VEGF₁₈₆. Grades of the tumor samples are indicated on the top of the lanes. See Fig. 1 for abbreviations.

Fig. 5. Structure of VEGF_{B155}. A. Nucleotide sequence and predicted amino acid sequence of VEGF_{B155}. B. Compared structures of VEGF_{B186}, VEGF_{B167}, and VEGF_{B155}. Splicing of exons 5 or 6 introduces a 1-base frameshift and a different reading frame. The stop codons of VEGF_{B167} and VEGF_{B155} are located 41 bases downstream of the stop codon of VEGF_{B186}.

novel form of VEGFB consisting of 155 amino acids. It lacks exons 5 and 6 and has the same C-terminal sequence as VEGFB₁₆₇ because splicing of exons 5 or 6 produces the same one-base frameshift. This form of VEGF is, however, a minor species and was detected only once.

Fig. 6. Regulation of VEGF mRNA and VEGFB mRNA by hypoxia. A. Human glioblastoma cells, rat C6 glioma cells, normal rat astrocytes, and rat brain capillary endothelial cells exposed for 6 h to either normoxic or hypoxic conditions. RNA was prepared and Northern blots were probed with VEGF, VEGFB, and GAPDH probes as indicated. B. Normalized VEGF/GAPDH signals in hypoxic cultures expressed relative to those observed in control cultures maintained under normoxic conditions. Means $\pm$ SEM ($n = 3$) are indicated. GBM, glioblastoma multiforme; ASTRO, astrocytes; EC, endothelial cells; C, control (normoxic) conditions; H, hypoxic conditions; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 7. Regulation of VEGF mRNA and VEGFB mRNA by dexamethasone. A. Northern blots showing the action of dexamethasone on VEGF and VEGFB expressions in cultured cells from three glioblastomas. Cells were incubated for 24 h in complete culture medium in the absence or presence of 1 μ M dexamethasone. RNA was isolated. Northern blots were probed with VEGF, VEGFB, and GAPDH probes as indicated. B. Normalized signals in dexamethasone-treated cultures expressed relative to those observed in control cultures. Means $\pm$ SEM ($n = 3$) are indicated. Dex, dexamethasone; C, cultured cells. See Fig. 6 for abbreviations.

The astrocytoma cells isolated from tumors and maintained in culture expressed VEGF mRNA and VEGFB mRNA. VEGF mRNA expression was enhanced by hypoxia, but the sensitivity of human and rat glioma cells to hypoxia was not greater than that of normal astrocytes. Thus, glioma cells were not sensitized to the lack of oxygen. VEGF expression has been reported to be inhibited by dexamethasone (Finkenzeller et al., 1995; Nauck et al., 1998). In this study, dexamethasone partially reduced VEGF mRNA expression by glioma cells. Clinically, glucocorticoids are used to reduce brain tumor-associated cerebral edema. Because VEGF is a vascular permeability factor (Leung et al., 1989), it can be surmised that a reduction of VEGF expression by glioblastoma cells can contribute to therapeutic action of glucocorticoids. Expression of VEGFB mRNA in glioblastoma cells grown in vitro is affected neither by hypoxia nor by dexamethasone. The lack of action of hypoxia may be due to the fact that the promoter of the *VEGFB* gene lacks hypoxia-sensitive

elements found in the promoter of the *VEGF* gene (Silins et al., 1997).

In conclusion, this study shows a surprising ubiquitous expression of VEGFB mRNA in human astrocytomas. Although this expression is not upregulated in high-grade astrocytomas and is insensitive to both hypoxia and glucocorticoids, the hypothesis that VEGFB contributes to the pathogenesis of astrocytomas cannot be excluded. VEGFB homodimers only recognize the VEGF receptor-1 (Flt1) (Olofsson et al., 1998). This receptor does not couple to the mitogen-activated protein kinase cascade and does not promote mitogenic actions. It probably plays a role in the degradation of the extracellular matrix, in cell migration, and in controlling vascular permeability (Seetharam et al., 1995). Heterodimers formed of VEGFB and VEGF are, however, mitogenic for endothelial cells, possibly by activating VEGF receptor-2 (Flk1/KDR) (Olofsson et al., 1998). The binding of VEGFB to Flt1 may also enable VEGF binding to VEGF receptor-2 (Flk1/KDR), and may thus promote mitogenic actions of VEGF.

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