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# Epidermal Growth Factor Stimulates Vascular Endothelial Growth Factor Production by Human Malignant Glioma Cells: A Model of Glioblastoma Multiforme Pathophysiology

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Hypervascularity, focal necrosis, persistent cerebral edema, and rapid cellular proliferation are key histopathologic features of *glioblastoma multiforme* (GBM), the most common and malignant of human brain tumors. By immunoperoxidase and immunofluorescence, we definitively have demonstrated the presence of vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFr) in five out of five human glioma cell lines (U-251MG, U-105MG, D-65MG, D-54MG, and CH-235MG) and in eight human GBM tumor surgical specimens. In vitro experiments with glioma cell lines revealed a consistent and reliable relation between EGFr activation and VEGF production; namely, EGF (1–20 ng/ml) stimulation of glioma cells resulted in a 25–125% increase in secretion of bioactive VEGF. Conditioned media (CM) prepared from EGF-stimulated glioma cell lines produced significant increases in cytosolic free intracellular concentrations of $Ca^{2+}$ ($[Ca^{2+}]_i$) in human umbilical vein endothelial cells (HUVECs). Neither EGF alone or CM from glioma cultures prepared in the absence of EGF induced $[Ca^{2+}]_i$ increases in HUVECs. Preincubation of glioma CM with A4.6.1, a monoclonal antibody to VEGF, completely abolished VEGF-mediated $[Ca^{2+}]_i$ transients in HUVECs. Likewise, induction by glioma-derived CM of von Willebrand factor release from HUVECs was completely blocked by A4.6.1 pretreatment. These observations provide a key link in understanding the basic cellular pathophysiology of GBM tumor angiogenesis, increased vascular permeability, and cellular proliferation. Specifically, EGF activation of EGFr expressed on glioma cells leads to enhanced secretion of VEGF by glioma cells. VEGF released by glioma cells in situ most likely accounts for pathognomonic histopathologic and clinical features of GBM tumors in patients, including striking tumor angiogenesis, increased cerebral edema and hypercoagulability manifesting as focal tumor necrosis, deep vein thrombosis, or pulmonary embolism.

## INTRODUCTION

It is an intriguing fact that *glioblastoma multiforme* (GBM), the most anaplastic of malignant brain tumors, is also the most common (>65%) of primary intracranial malignant tumors in adults. In almost every biochemical, immunological, morphological, and biological category examined, GBM tumors exhibit striking heterogeneity (Bigner *et al.*, 1981; Wikstrand *et al.*, 1985). The degree of heterogeneity associated with these tumors has made it difficult to unravel the molecular and cellular mechanisms underlying GBM pathogenesis.

Histopathologically, GBM are characterized by striking pleomorphism in cellular and nuclear detail with pathognomonic features of focal necrosis and neovascularization. It is the appearance of necrotic foci that

shifts the diagnosis from anaplastic astrocytoma (grade III) to GBM (grade IV) (Fulling and Nelson, 1984; Burger and Kleihues, 1989), which has a documented poorer prognosis (Burger and Green, 1987). Other characteristic histopathological features of this uniformly fatal brain tumor relevant to this report include marked endothelial cell proliferation and increased vascular permeability. Biochemical analyses of tissue culture media conditioned either by GBM tumor explants or U-251MG, an established glioma cell line, indicated the presence of vascular permeability factor (VPF)-like activity (Criscuolo *et al.*, 1989).

VPF, also known as vascular endothelial cell growth factor (VEGF), is a potent and specific endothelial cell mitogen in vitro (Leung *et al.*, 1989) and induces angiogenesis in vivo (Conn *et al.*, 1990). VEGF is 1000-fold more potent than histamine in inducing capillary permeability (Connolly, 1991). Furthermore, VEGF increases von Willebrand Factor (vWF) release (Brock *et al.*, 1991) and thromboplastin activity (Clauss *et al.*, 1990) in human endothelial cells, two physiological changes that could enhance coagulation. Taken together, these important biological activities indicate that VEGF may play major roles in the angiogenesis and increased edema characteristic of malignant gliomas and may contribute to hypercoagulability in these patients (Bostrom *et al.*, 1986).

Recent studies indicate that almost all malignant glioma cells and cell lines express the receptor for epidermal growth factor (EGFr), and the majority of GBM tumors show amplification or rearrangement of the EGFr gene (Sang *et al.*, 1989; Blumenstock *et al.*, 1991; Maruno *et al.*, 1991). Furthermore, Maruno *et al.* (1991) have shown an increased proliferative index for EGFr-positive malignant glioma cells as compared with glioma cells that do not express this receptor. The implication is that activation of EGFr may play a role in the persistent proliferation of GBM. EGF is a low molecular weight (6 kDa) growth factor that is a normal constituent of human plasma (Tuomela, 1990) and is mitogenic for cells expressing EGFr. Several groups also have demonstrated that some gliomas show increased mRNA levels and express transforming growth factor-$\alpha$ (TGF-$\alpha$), which can activate EGFr in vitro (Maxwell *et al.*, 1991) in an equimolar fashion equivalent to EGF. Therefore, the ligand(s) responsible for activating EGFr, i.e., EGF and TGF-$\alpha$, are either constitutively present in plasma and plasma ultrafiltrates or can be produced by glioma cells themselves.

Until now, there has been no demonstrated relation between EGFr expression in gliomas and associated vascularity of these tumors. In this study we demonstrate the simultaneous expression of VEGF and EGFr in five out of five brain tumor cell lines examined. In eight postoperative surgical specimens, both VEGF and EGFr were determined by immunohistochemical means to be variably expressed. More importantly, we demonstrate for the first time that activation of EGFr by EGF leads to increased secretion of bioactive VEGF by malignant glioma cells. This physiological relation provides a useful paradigm to explain the paradoxical existence of intense neovascularity and necrotic foci in GBM tumors.

## MATERIALS AND METHODS

### *Biochemical and Biological Reagents*

Dulbecco's modified Eagle's medium mixed 1:1 with Ham's F-12 (DMEM/F12; Sigma Chemical, St. Louis, MO) and MCDB 105 (Sigma) supplemented to 2 mM with L-glutamine were used for culture of all glioma cells. Medium 199 (M199; GIBCO, Grand Island, NY) was used as the culture medium for human umbilical vein endothelial cells (HUVEC). Fetal bovine serum (FBS) and calf serum (Hyclone, Logan, UT) were heat inactivated (56°C, 45 min). EGF (human, receptor grade; Collaborative Research, Bedford, MA), dexamethasone (Sigma), $\beta$-phorbol (Sigma), and phorbol myristate acetate (PMA; Sigma) were all diluted at specified concentrations in Dulbecco's phosphate-buffered saline (PBS, pH 7.2). Transforming growth factor-$\beta$1 (TGF-$\beta$1) (R&D Systems, Minneapolis, MN) was prepared as 1 $\mu$g/ml stock solution by dilution in exipient (25% isopropyl alcohol + 0.1% trifluoracetic acid). Further dilution was carried out in PBS containing 3 mM CHAPS (Boehringer Mannheim, Mannheim, Germany). Recombinant VEGF (rhVEGF; 165 amino acid isoform) was purified at Genentech (South San Francisco, CA). Purified guinea pig VPF was used as a positive control for vWF assay and was obtained as a generous gift from Dr. Donald Senger (Beth Israel Hospital, Boston, MA). Fura-2/AM was purchased from Molecular Probes (Junction City, OR). Culture media and sera were determined to have <100 pg/ml of endotoxin by the Limulus amoebocyte lysate assay.

### *Cell Lines*

GBM cell lines U-251MG, U105MG, D-65MG, and D-54MG were obtained as a gift from Dr. Darell D. Bigner (Duke University, Durham, NC) and have been described in detail (Ponten and MacIntyre, 1968; Ponten and Westermark, 1978; Bigner *et al.*, 1981). CH-235MG was cultured from a portion of a GBM tumor removed in 1979 from a 60-year-old female and recently has been characterized (Bethea *et al.*, 1992a). Karyotypic analyses of these cell lines have revealed that each is karyotypically unique with complex numerical and structural deviations. Karyotypes for U-251MG, U105MG, D-65MG, and D-54MG were similar to those previously reported (Bigner and Mark, 1984; Bigner *et al.*, 1985). Moreover, restriction fragment length polymorphism mapping profiles produced by restricting cell line DNA with *Hae* III and probing Southern blots with YNH24 (locus D2S44, 0.96 polymorphic discrimination) and pH30 (locus D4S139, 0.99 polymorphic discrimination) were nonidentical, corroborating that these tumor cell lines originated from different patients. All of the glioma cell lines have been passaged >40 times, and, for experiments, the cell lines were maintained in 150-cm$^2$ plastic tissue culture flasks (Falcon Plastics, Lincoln Park, NJ) in complete culture media (DMEM/F12 + 2 mM L-glutamine + 8% FBS) at 37°C and 7.5% $CO_2$. Antibiotics were not used routinely, and all cell lines were found by routine testing to be negative for mycoplasma by Hoechst 33258 stain (Chen, 1977). Cells were grown to postconfluence with medium changes every third day and were harvested using brief incubation (10 min, room temperature [RT] in 7 ml 0.05% Trypsin/0.53 mM EDTA solution [GIBCO]. FBS (2 ml) was added to the flask to inactivate the trypsin, and adherent cells were released by trituration, pelleted by centrifugation (200 × *g*, 8 min, 22°C), and resuspended in 10 ml of DMEM/F12. Viable cell counts were determined by trypan blue exclusion.

### *Human Endothelial Cell Cultures*

Primary cultures of human endothelial cells (HUVECs) were established from pooled umbilical cords and were propagated (passages

2–5) as previously described (Brock and Capasso, 1988). Medium 199 was supplemented with 15% FBS, 10 μM thymidine (Sigma), 100 μg/ml porcine heparin (Sigma), 50 μg/ml endothelial cell growth factor (Biomedical Technologies, Stoughton, MA), 100 μg/ml of streptomycin, and 100 U/ml of penicillin (GIBCO). HUVECs were serially passaged twice per week by harvesting with trypsin/EDTA and seeding at a 1:4 ratio into gelatin (0.1%)-coated 75-$cm^2$ flasks. For these experiments, HUVECs between passage levels 2 and 6 were seeded into 35- or 100-mm gelatin-coated dishes, medium was exchanged every other day, and the cells were used after 6–8 d.

## *VEGF Secretion by U-105MG and U-251MG Cell Lines In Vitro*

Glioma cells were plated at $2.5 \times 10^5$ cells/well in 24-well microtrays (Costar 3524, Costar, Cambridge, MA) in 400 μl of DMEM/F12 + 2 mM L-glutamine + 10% FBS, and cells were incubated for 3 d at 37°C in 7.5% $CO_2$. Monolayers were rinsed once with serum-free MCDB 105 medium, and 400 μl of fresh MCDB 105 + 2 mM L-glutamine containing 0, 2, 5, or 10% FBS was added to triplicate wells of each plate. At 24-h intervals, the medium and cell monolayers in one microplate were harvested as follows. Medium from each well was clarified by centrifugation ($3000 \times g$, 15 min, 4°C) in 1.5-ml polypropylene microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA). Individual aliquots (350 μl) were stored frozen (−20°C) in separate microcentrifuge tubes until analyzed for VEGF by enzyme-linked immunosorbent assay (ELISA). Glioma cells were harvested from monolayer cultures with 1 or 2 ml of trypsin/EDTA solution, and viable cell numbers were determined by trypan blue exclusion.

## *Growth Factor and Pharmacologic Stimulation of Glioma Cell Lines*

The effect of specific stimuli on VEGF secretion was assessed in glioma cell lines U-251MG, U-105MG, D-65MG, and D-54MG. Each glioma cell line was plated at $3 \times 10^5$ cells/well in 6-well plates (no. 3407; Falcon) in 2 ml of complete culture medium and incubated (37°C, 7.5% $CO_2$) undisturbed for 72 h. Monolayers were rinsed once with serum-free DMEM/F12 and 1 ml of serum-free DMEM/F12 containing either recombinant human EGF (20 ng/ml), TGF-β1 (1 ng/ml), dexamethasone (200 ng/ml), PMA (160 nM), β-phorbol (160 nM), or PBS was added to replicate wells. After a 5-d incubation (37°C, 7.5% $CO_2$), the conditioned media (CM) were collected, clarified by centrifugation, transferred to clean polypropylene microcentrifuge tubes, and held frozen (−20°C) until assayed for VEGF by ELISA.

Various EGF concentrations were used to induce VEGF in the U-105MG cell line as follows. U-105MG monolayers were established in 24-well plates by incubating (72 h, 37°C) $2.5 \times 10^5$ cells/well in 400 μl of DMEM/F12 containing 10% FBS. Monolayers were rinsed once with serum-free medium, and 400 μl of DMEM/F12 containing 3% FBS was added to each well. EGF (0–30 ng/ml) or PBS was added (10 μl) to triplicate wells. After incubation (120 h, 37°C, 7.5% $CO_2$), media from individual wells were harvested separately. Clarified supernates were stored frozen (−20°C) until VEGF levels were quantified by ELISA.

## *Sandwich ELISA for VEGF*

Ninety-six-well microtiter plates (Maxisorb; Nunc, Kamstrup, Denmark) were coated with monoclonal antibody (mAb) 4.6.1 (Kim *et al.*, 1992) by incubating overnight at 4°C with 100 μl/well of antibody at 2.5 μg/ml in 50 mmol/l of sodium carbonate buffer, pH 9.6 (coat buffer). After removal of the coating solution, the coated plates were blocked with 150 μl/well of 5% BSA in PBS for 1 h at RT and washed six times with 0.5% Tween 20 in PBS (wash buffer).

Standards were freshly prepared by dilution of rhVEGF (1 mg/ml established by quantitative amino acid analysis) with assay buffer (PBS containing 5% BSA, 0.05% Tween 20, and 0.01% Thimerosal) to 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 ng/ml. Diluted standards and samples were dispensed onto coated wells (100 μl/well), and plates were sealed and incubated at room temperature for 2 h with gentle agitation. Horseradish peroxidase (HRPO) labeled mAb 3.13.1 (Kim *et al.*, 1992) was then added (100 μl/well). After further incubation (1 h, RT), the plates were washed. Freshly prepared OPD substrate solution (0.4 g of o-phenylenediamine dihydrochloride/l of PBS plus 0.4 ml of 30% hydrogen peroxide) was added (100 μl/well) and incubation (15 min, RT) carried out in the dark. The reaction was stopped by the addition of 100 μl of 2.25 mol/l sulfuric acid and absorbance at 490 nm determined on a $V_{max}$ plate reader (Molecular Devices, Menlo Park, CA). A standard curve was generated by plotting absorbance vs. log of rhVEGF concentration, using a four-parameter nonlinear regression curve fitting program (developed at Genentech). Sample concentrations were obtained by interpolation of their absorbance on the standard curve.

## *Bulk Production of Glioma Cell Line Conditioned Medium*

U-251MG or U-105MG glioma cell lines were grown to confluence (72 h, 37°C) in 225-$cm^2$ flasks in DMEM/F12 containing 10% FBS. Cell monolayers were washed twice with serum-free DMEM/F12, and 35 ml of serum-free medium containing 20 ng/ml of EGF or PBS were added to each flask and incubated undisturbed for 72 (U-105MG) or 96 h (U-251MG). This CM was collected, clarified by centrifugation ($800 \times g$, 10 min, RT), and concentrated with extensive dialysis against PBS (Centriprep-10; Amicon Division, Beverly, MA). Final concentration was achieved with Centricon-10 microconcentrators (Amicon). Total volume reduction for U-251MG CM was 70-fold, and for U-105MG CM was 84.5-fold. A 150-μl aliquot of each concentrate was stored at 4°C before calcium flux assay, and the remainder was stored at −20°C before quantification of VEGF by ELISA.

## *Measurement of $Ca^{2+}$-Sensitive Fura-2 Fluorescence*

Fura-2, a $Ca^{2+}$-sensitive fluorescent dye (Grynkiewicz *et al.*, 1985), was used to monitor changes in cytosolic free intracellular calcium concentration ($[Ca^{2+}]_i$) in HUVEC suspensions as described by Brock and Cappasso (1988). For these experiments, HUVEC were grown on gelatin (0.1%)-coated 100 mm (diam) dishes. To detach cells, six to eight dishes ($\sim 1.5–3 \times 10^7$ cells) were briefly exposed to Hank's balanced salt solution without calcium or magnesium and with 2 mM EDTA and 0.1% BSA. Cells were pelleted by centrifugation ($200 \times g$, 3 min) and resuspended in serum-free M199 ($5 \times 10^6$ cells/ml). The cell suspension was incubated with fura-2/AM (0.1 μM, 20 min, 37°C) and then diluted tenfold with M199 and incubated for an additional 10 min. Cells were pelleted by low-speed centrifugation, resuspended in M199 (with 1.5 mM $CaCl_2$), pelleted again, and finally resuspended ($4 \times 10^5$ cells/ml) in medium 199 containing 1% BSA. The cell suspension was divided into 1-ml aliquots in microcentrifuge tubes and kept at 8–10°C. Before fluorescence measurements, HUVEC suspensions were equilibrated for 10 min at 37°C, spun in an Eppendorf microcentrifuge, resuspended in 1.6 ml warm balanced salt solution (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, buffered to pH 7.4 with tris(hydroxymethyl)aminomethane base), and placed in a cuvette. Cell viability was >95% by trypan blue exclusion.

Fluorescence measurements were made using a SPEX Fluorlog II spectrofluorimeter (model ARCM; SPEX Industries, Edison, NJ) equipped with a stirring apparatus and thermostatted cuvette holder. This instrument has a beam splitter, dual filters, and chopping mechanism to allow rapid alternating excitation of fura-2 in $Ca^{2+}$-bound (340 nm) and $Ca^{2+}$-free (380 nm) states. The ratio of emitted fluorescence signals (505 nm) permits the calculation of $[Ca^{2+}]_i$ within the cells. $[Ca^{2+}]_i$ is calculated using the formula $[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R) \times (EGTA_{380}/DIG_{380})$, where R is the fluorescence ratio within the cuvette, $K_d$ is 224 nM, $EGTA_{380}$ is the fluorescence signal (380 nm) in the presence of 10 mM EGTA (pH 10), and $DIG_{380}$ is the

fluorescence signal following digitonin addition in the presence of 1.5 mM $CaCl_2$. Fluorescence tracings depicting $[Ca^{2+}]_i$ recording are typical tracings representative of duplicate experiments.

### *Sandwich ELISA for vWF*

Ninety-six-well microtiter plates (Maxisorb; Nunc) were coated with goat $F(ab)'_2$ anti-vWF (American Diagnostica, Greenwich, CT) by incubating overnight at RT with 100 μl/well of antibody at 2 μg/ml in 50 mmol/l of sodium carbonate buffer, pH 9.6 (coat buffer). After removal of the coating solution, coated plates were blocked with 200 μl/well of 5% BSA in PBS (1 hr, RT), and washed four times with wash buffer.

Standards were freshly prepared by diluting human vWF with assay buffer (PBS containing 5% BSA, 0.05% Tween 20) to 6.25, 3.12, 1.56, 0.78, and 0.39 mU/ml. The diluted standards and samples were dispensed onto the coated wells (100 μl/well). Plates were sealed and incubated at room temperature for 2 h with gentle agitation. Plates were washed as above and HRPO-labeled goat Fab anti-vWF was then added (100 μl/well). After further incubation (1 h, RT), the plates were washed, freshly prepared OPD substrate solution was added (100 μl/well), and incubation was carried out in the dark (15 min, RT). Enzymatic reaction was stopped by addition of 2.25 M sulfuric acid (100 μl/well) and absorbance (490 nm) determined using a microtiter plate reader (model 450; Bio-Rad, Richmond, CA). A standard curve was generated by plotting absorbance vs. vWF concentration; sample concentrations were obtained by interpolation of their absorbance relative to standards.

### *Immunohistochemistry/Immunofluorescence*

Immunohistochemical staining for VEGF or EGFr was performed on glioma cell lines U-251MG, U-105MG, CH-235MG, and D-65MG or on eight different GBM tumors obtained at surgery. Freshly resected GBM tumors were debrided, blocked, and frozen in O.C.T. mounting medium (Miles Laboratories, Naperville, IL). Frozen sections (10 μm thick) of GBM tumors that were mounted on poly-L-lysine coated slides or saline-rinsed confluent glioma cell line monolayers grown on glass slides (8-place Lab-Tek [Naperville, IL] culture chambers) were immersed in ice-cold 100% methanol for 30 s and allowed to dry. Slides were incubated in 3% BSA/PBS (30 min, RT) to block nonspecific binding, and excess blocking solution was replaced by a primary antibody solution in 3% BSA/PBS containing either 10 μg/ml of A.4.6.1 (anti-VEGF), 10 μg/ml of Mab 425 (anti-EGFr) (Murthy *et al.*, 1987), 1:2000 dilution of 29.1.1 (anti-EGFr ascites; Sigma), or a phycoerythrin-conjugated control polyclonal mouse IgG fraction (Becton Dickinson, Mountain View, CA). Glioma tissue sections or glioma cell line monolayers were incubated overnight in a humid atmosphere at RT and then extensively rinsed with PBS. For fluorescence staining, the slides were incubated (1 h, RT) with rabbit FITC-conjugated anti-mouse IgG (Southern Biotechnology, Birmingham, AL) at 10 μg/ml in 3% BSA/PBS and then rinsed three times with PBS. For immunoperoxidase staining, the manufacturer's instructions were followed as described for the Vectastain Elite ABC IgG kit (Vector Laboratories, Burlingame, CA). All slides were coverslipped with 10% polyvinyl alcohol.

Alternatively, we have used the antigen retrieval system (BioGenex, San Ramon, CA) to demonstrate immunoperoxidase staining in formalin-fixed paraffin-embedded specimens of GBM tissues obtained through the UAB Tissue Procurement Facility (University of Alabama, Birmingham). Histopathological diagnoses were provided by UAB neuropathologists or outside consultants. Paraffin tissue sections, cut at 10 μm, were mounted on TEPSA (3-aminopropyltriethoxysilane; Aldrich Chemical, Milwaukee, WI)-coated slides and baked (1 h, 58°C). Mounted sections were soaked for 3 min each in two changes of xylene for deparaffinization, then dehydrated in graded changes of ethanol (70, 95, 100, and 100%) and rehydrated in PBS. Endogenous peroxidase was blocked by soaking mounted sections in 1.5% $H_2O_2$ in methanol. Each slide was then washed in deionized water and processed according to manufacturer's instructions for the antigen retrieval system. Nonspecific binding was blocked by incubating sections in 5% BSA/PBS for 45–60 min. mAbs A.4.6.1 (α-VEGF) or OKT4 (α-CD4; hybridoma obtained from American Type Culture Collection, Rockville, MD) were diluted in 5% BSA/PBS, and each section was incubated with one of the mAbs (100 μl, 1 h, 37°C). Tissue sections were washed in two changes of 1% Triton-X100/PBS for 10 min each, followed by two rinses with PBS. Vectastain Elite ABC kit was used as described above to complete the immunoperoxidase staining. Tissue sections were counterstained with aqueous hematoxylin and coverslips mounted with Permount (Fisher Scientific, Pittsburgh, PA).

## RESULTS

### *Glioma Cell-Derived VEGF*

VEGF was detected at various concentrations in the CM of U-251MG, U-105MG, D-65MG, and D-54MG glioma cell lines as detected by a VEGF-specific ELISA. VEGF accumulation in U-251MG conditioned medium

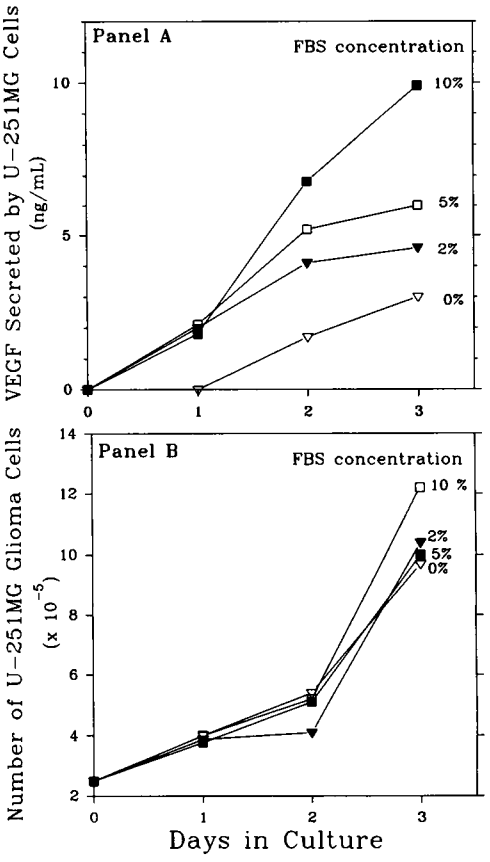


**Figure 1.** VEGF production by human glioma cell lines is enhanced in a dose-dependent fashion by fetal bovine serum component(s). (A) VEGF secretion by U-251MG glioma cells increased with time in culture and was enhanced in a dose-dependent fashion by increasing concentrations of FBS. (B) U-251MG glioma cells proliferated comparably in 0–5% FBS and only slightly faster in 10% FBS, thus indicating that cell numbers alone did not provide a trivial explanation for the FBS-induced increase in VEGF production.

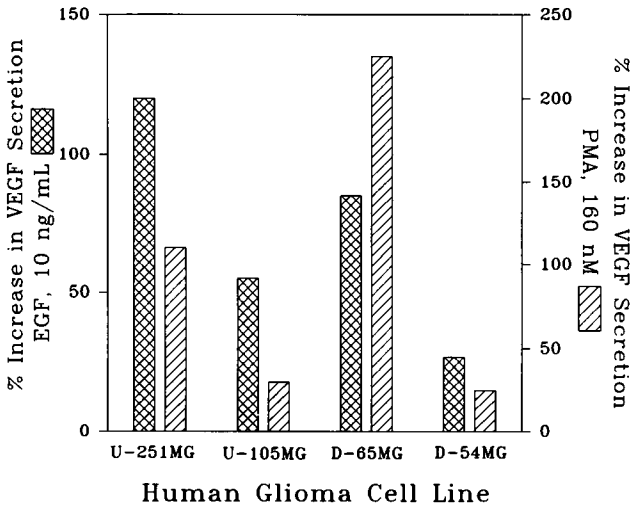


**Figure 2.** Ability of stimuli to induce VEGF secretion by human glioma cell lines. PMA at 160 nM and EGF at 10 ng/ml enhanced VEGF secretion by each of the four glioma cell lines examined. VEGF in culture supernates was quantified by ELISA, and percentage increases were calculated in comparison to stimulation by $\beta$-phorbol (for PMA) or PBS (for EGF).

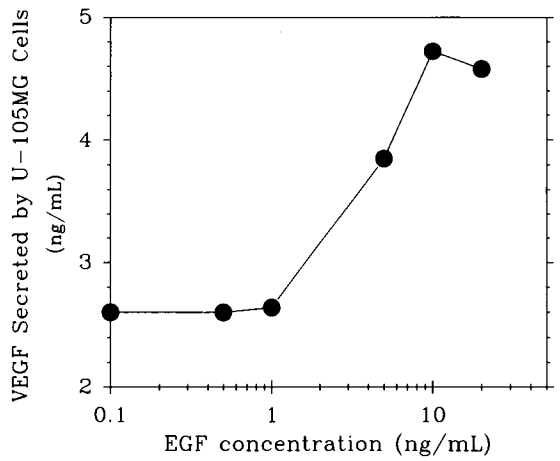


**Figure 3.** EGF induced VEGF secretion by U-105MG glioma cells in a dose-dependent fashion with maximal stimulation at 10 ng/ml. VEGF in 96-h glioma cell CM was quantified by ELISA.

(Figure 1A) correlated positively with increasing FBS concentrations (0–10%). Assays performed with diluted FBS indicated that culture medium not exposed to cells did not have detectable immunoreactive VEGF. The mean concentration of VEGF in medium from glioma cells (initial density $2.5 \times 10^5$/well) grown in serum-free conditions averaged 2.3 ng/ml on day 3. In contrast, the CM from cells grown in 10% FBS contained an average VEGF concentration of 10.2 ng/ml by day 3. This represented a 340% increase in the amount of VEGF produced by these cells. To determine whether this increase reflected an enhanced VEGF production or was due to improved cell survival and proliferation, we concurrently counted viable cells trypsinized from the same wells from which the supernates were harvested. Growth curves (Figure 1B) of U-251MG cells in medium containing varying FBS concentrations revealed that, by day 3, cell proliferation was highest in 10% FBS ($1.23 \times 10^6$ cells/well) as compared with that of cells grown in the serum-free medium ($9.8 \times 10^5$ cells/well). However, this only represented a 22% increase in cell number. These data indicated that modest increases in glioma cell numbers did not fully account for increased VEGF production; rather, a significant portion of the threefold increase in VEGF appeared to be due to *increased secretion*. Similar comparisons for the U-105MG cell line yielded qualitatively similar results.

### *Stimulation of VEGF by EGF*

Induction of VEGF secretion by various stimuli was examined using four glioma cell lines maintained in serum-free media. Both PMA (160 nM; Figure 2) and EGF (20 ng/ml; Figure 2) stimulated VEGF secretion in all four cell lines when compared with that induced by either beta phorbol (160 nM) or PBS. Increased VEGF secretion by U-105MG was induced by EGF at physiological concentrations of 1–20 ng/ml (Figure 3). VEGF secretion was not consistently suppressed or enhanced after administration of dexamethasone or TGF-$\beta$1 to glioma cells (Table 1).

### *Functional Activities of Glioma-Derived VEGF*

Although ELISA can provide a very accurate estimate of immunoreactive VEGF, biological activity of VEGF could not be determined using this assay. Therefore, we assayed changes of $[Ca^{2+}]_i$ within, and vWF secretion by, HUVECs as a measure of VEGF activity produced by cultured human glioma cells.

**Table 1.** Effect of dexamethasone or TFG-$\beta$ on secretion of VEGF by glioma cell lines

| Glioma cell line | Unstimulated secretion (ng/ml) | Percent change in VEGF secretion[a] | |
|---|---|---|---|
| | | Dexamethasone (2 mM) | TGF-$\beta$1 (2 ng/ml) |
| U-251MG | 3.84 | +116[b] | −20[b] |
| U-105MG | 6.22 | −20 | −40 |
| D-65MG | 1.02 | ND[c] | +77 |
| D-54MG | 3.97 | +56 | +15 |

[a] VEGF secretion measured by ELISA in 72–96 h CM.
[b] Values are percents.
[c] ND, not detectable.

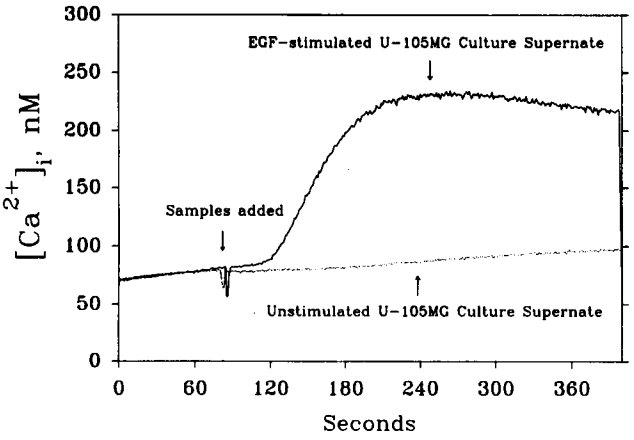


**Figure 4.** Concentrated CM (16 μl) from EGF-stimulated U-105MG glioma cells induced an unequivocal increase in $[Ca^{2+}]_i$ in HUVECs. In comparison, comparable CM derived from U-105MG glioma cells that were not exposed to EGF did not induce $[Ca^{2+}]_i$.

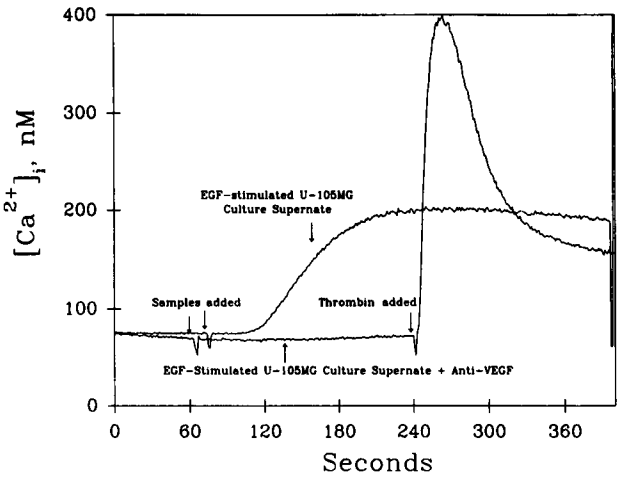


**Figure 5.** The $[Ca^{2+}]_i$ transient in HUVECs induced by EGF stimulated U-105MG glioma cell CM was abolished by preincubation of CM with 1 μg/ml of anti-VEGF antibody A4.6.1. In contrast, A4.6.1. did not affect the thrombin- (1 U/ml) induced $[Ca^{2+}]_i$ transient.

**Changes in $[Ca^{2+}]_i$.** As illustrated in Figure 4, CM obtained from U-105MG glioma cells stimulated for 72 h with EGF induced an increase in $[Ca^{2+}]_i$ in HUVEC suspensions. Furthermore, this effect was not obtained unless the glioma cells were stimulated with EGF; EGF itself did not alter HUVEC $[Ca^{2+}]_i$. Qualitatively similar fluorescence tracings were obtained using supernates prepared in an identical fashion from U-251MG cell line cultures.

To confirm that VEGF in glioma CM was responsible for the $[Ca^{2+}]_i$ increase, we used an anti-VEGF monoclonal antibody to neutralize VEGF (Kim *et al.*, 1992). As demonstrated in Figure 5, mAb A.4.6.1 completely inhibited the ability of EGF-induced glioma CM to increase $[Ca^{2+}]_i$ in HUVECs. Addition of an antigen irrelevant monoclonal antibody (α-S100 protein; $IgG_{2a}$) was without effect, yielding a transient similar to that of medium only. Thrombin (1 U/ml) was added at 240 s to demonstrate that the lack of VEGF-induced $[Ca^{2+}]_i$ change was not due to a nonspecific effect of the antibody. These experiments confirmed that alterations in $[Ca^{2+}]_i$ were specifically due to VEGF in glioma CM.

**vWF Secretion.** As previously described (Brock *et al.*, 1991), another biological activity of VEGF is its ability to stimulate release of vWF from HUVECs. Enhanced release of vWF was induced by EGF-stimulated glioma cell CM (Figure 6). Again, as demonstrated above, vWF release was blocked by anti-VEGF mAb A.4.6.1 but not by an antigen irrelevant antibody specific for S-100 protein. As previously described (Brock *et al.*, 1991), both thrombin and purified VEGF were effective stimulants for vWF release in HUVECs.

### *Immunohistochemical Staining*

The presence of VEGF in frozen sections of two human GBM surgical specimens was demonstrated using both fluorescein- (Figure 7, left) and peroxidase-labeled immunohistochemical staining methods. Both methods produced similar results; namely, a diffuse cytoplasmic and diffuse pericellular staining pattern. Normal brain grey matter from several patients undergoing routine temporal lobectomy for intractable seizures was consistently negative. Attempts to use the A.4.6.1 mAb for

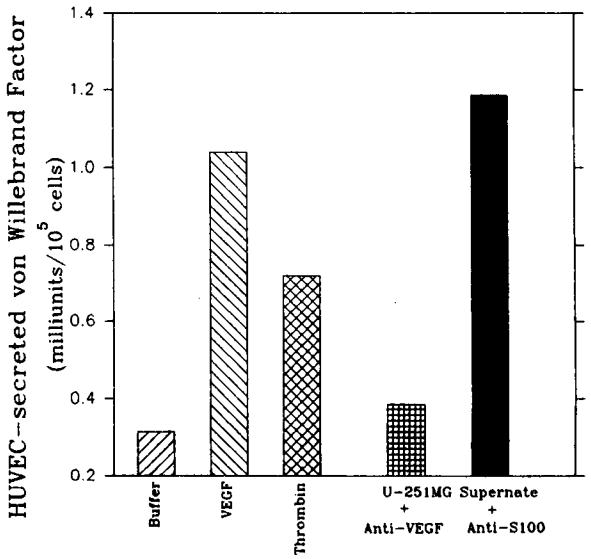


**Figure 6.** Stimulation of vWF secretion by HUVECs. Both VEGF (100 ng/ml; left-diagonal striped bar) and thrombin (3 U/ml; diagonal cross-hatched bar) induced ELISA-quantified vWF secretion by HUVECs compared with nonstimulated glioma cells (right-diagonal striped bar). VEGF in U-251MG glioma cell line CM was neutralized by 1 μg/ml of A4.6.1 anti-VEGF monoclonal antibody (horizontal cross-hatched bar) but not by 1 μg/ml of 15E2E2 anti-S100 protein monoclonal antibody (solid bar).

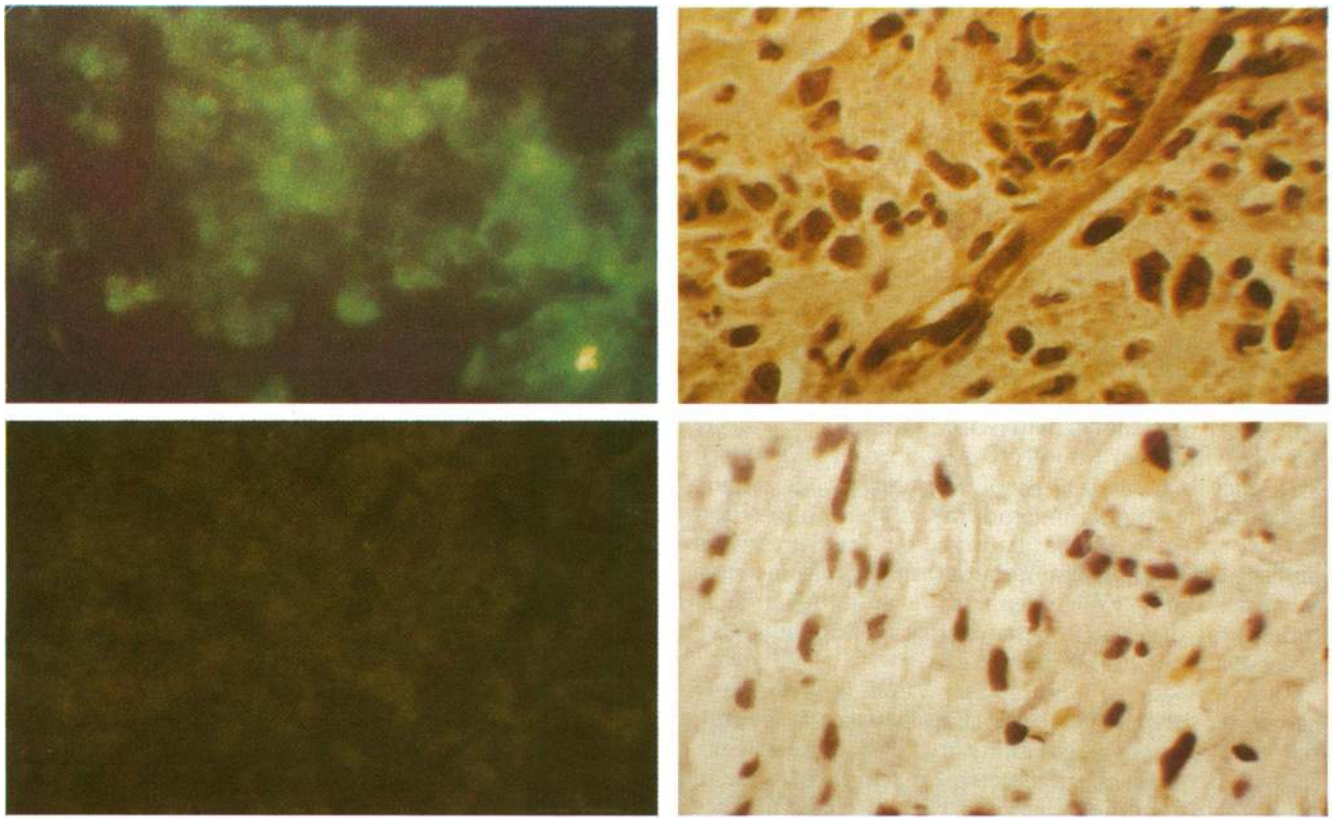

**Figure 7.** Methanol-fixed frozen sections of a GBM tumor showed a diffuse cytoplasmic staining pattern with anti-VEGF antibody A4.6.1 and FITC-labeled rabbit anti-mouse IgG (upper left). Polyclonal mouse IgG1 did not bind as assessed with FITC-rabbit anti-mouse IgG (lower left). Binding of A4.6.1 in paraffin sections of GBM tissue (upper right) was observed by immunoperoxidase staining after antigen retrieval processing; anti-CD4 monoclonal mouse (IgG1) antibody was not reactive (lower right). Paraffin sections were counterstained with aqueous hematoxylin.

staining formalin-fixed paraffin-embedded tissue sections were initially unsuccessful. However, when six archival GBM tissue sections were processed using an antigen retrieval system (BioGenex), we could reliably and reproducibly identify VEGF in all six of these human brain tumor sections (Figure 7, right). Again, normal cerebral cortex did not stain with this antibody. To date, we have demonstrated VEGF by various immunohistochemical methods in all eight human GBM tumor specimens examined (Table 2).

EGFr were also detected in two of three human GBMs examined. mAb 29.1.1 is able to recognize EGFr in human formalin-fixed paraffin-embedded GBM tissue sections without using the antigen retrieval system; furthermore, its application in this instance was not helpful in improving the strength or pattern of reactivity that was observed throughout the cytoplasm of the tumor cells, as well as localized to their surfaces (Figure 8). All four glioma cell lines examined (U-251MG, D-65MG, D-54MG, and CH-235MG) stained positively for VEGF (Figure 9, A and B) and EGFr (Figure 10, A and B). Anti-VEGF staining of glioma cell lines revealed diffuse cytoplasmic distribution with focally intense perinuclear staining and a delicate cell surface localization. Anti-EGFr stained coarsely in clumps on cell surfaces of glioma cell lines with diffuse cytoplasmic staining that was less dense than that of intracytoplasmic VEGF staining. A polyclonal mouse $IgG_1$ fraction used as the first antibody did not react with glioma cell lines.

Of the five glioma cell lines examined, we have detected production of VEGF by ELISA, immunohistochemistry, or calcium mobilization assays in all five (Table 3).

## DISCUSSION

Angiogenesis plays a critical role in tumor progression by providing oxygen and nutrients to rapidly dividing tumor cells and allowing for rapid elimination of $CO_2$ and waste metabolites. Folkman (1972) initially hypothesized that tumors liberate specific substances that stimulate and maintain a continued blood supply to the tumor bed. Since then, numerous angiogenic substances have been identified, including fibroblast growth factor

(FGF) (Gospodarowicz *et al.*, 1987), TGF-$\alpha$ (Schreiber *et al.*, 1986), TGF-$\beta$ (Roberts *et al.*, 1986), angiogenin (Fett *et al.*, 1985), platelet-derived growth factor (PDGF) (Bar *et al.*, 1989), platelet derived-endothelial cell growth factor (PD-ECGF) (Ishikawa *et al.*, 1989), and VEGF (Connolly, 1991; Ferrara *et al.*, 1991). Until now, the role of any particular angiogenic factor in pathogenic development of primary malignant glial neoplasms tumors has not been clearly defined.

Histologically, GBM tumors exhibit characteristic morphological features of endothelial cell proliferation and leaky vascularity that undoubtedly contribute to peritumoral edema that is a consistent finding associated with these neoplasms. Moreover, a small but significant proportion of patients with GBM tumors, as well as patients with other tumor types (Monreal *et al.*, 1991), experience an increased risk of thromboembolic events. In addition to stimulating endothelial cell proliferation in vitro and neovascularity in vivo, VEGF increases capillary permeability, stimulates thromboplastin activity, and induces vWF release from endothelial cells. None of the other angiogenic factors mentioned above possesses all of these capabilities.

Thus, it is our contention that VEGF is most likely the central mediator of angiogenesis, vascular permeability, and hypercoagulability in GBM tumors. Correlations between specific biological activities of VEGF and both histopathological and clinical characteristics of GBM tumors provide presumptive evidence that VEGF plays a significant role in GBM tumor vessel pathophysiology. This presumption is conclusively established by our demonstration of bioactive VEGF secretion from all human glioma cell lines examined, as well as a immunohistochemical localization of VEGF in an intra- and pericellular distribution of VEGF.

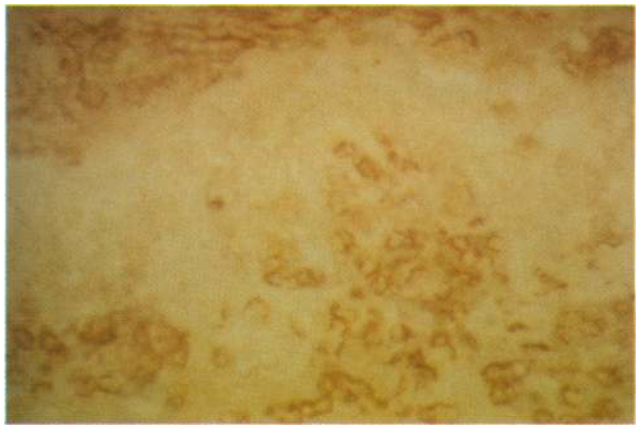

**Figure 8.** EGF receptors in formalin-fixed paraffin-embedded sections from a GBM tumor sections. Monoclonal antibody 29.1.1 reacted positively with cell surface EGFr as detected by indirect immunoperoxidase staining techniques.

We have demonstrated for the first time immunohistochemical staining of VEGF in human GBM surgical specimens and human GBM cell lines. In all glioma cell lines examined, VEGF could be demonstrated intracellularly by immunohistochemistry and, as a secreted product as detected by immunohistochemistry, ELISA and bioassay. Further evidence supporting that VEGF is secreted by glioma cells derives from the fact that human VEGF mRNA for the precursor encodes a hydrophobic signal sequence. This is in contrast to acidic

**Table 2.** Immunohistochemical demonstration of VEGF and EGFr in malignant gliomas

| | | Immunofluorescence results | | Immunoperoxidase results | |
|---|---|---|---|---|---|
| Tumor tissue designation | Diagnosis | VEGF | EGFr | VEGF | EGFr |
| Frozen sections | | | | | |
| 90-112141 | GBM | ++ | ++ | + | ++ |
| 92-001461 | GBM | + | + | + | ND[a] |
| Archival paraffin sections | | | | | |
| 89-006119 | GBM | ND | ND | +++ | ND |
| 90-003221 | GBM | ND | ND | ND | ++ |
| 91-06A011 | GBM | ND | ND | ++ | ND |
| 91-10A149B | GBM | ND | ND | + | ND |
| 92-06A102 | GBM | ND | ND | +++ | ND |
| 92-07A002 | GBM | ND | ND | ++++ | 0 |
| 92-08A182 | GBM | ND | ND | +++ | ND |
| Total Tumors Positive/Total tested | | 2/2 | 2/2 | 8/8 | 2/3 |

Frozen sections tested by both indirect immunofluorescence and indirect immunoperoxidase; paraffin sections tested after antigen retrieval by indirect immunoperoxidase only; scoring based on intensity with 0, negative; +, light positive staining; ++, moderate staining; +++, strong positive staining; ++++, intense positive staining.

[a] ND = not done.

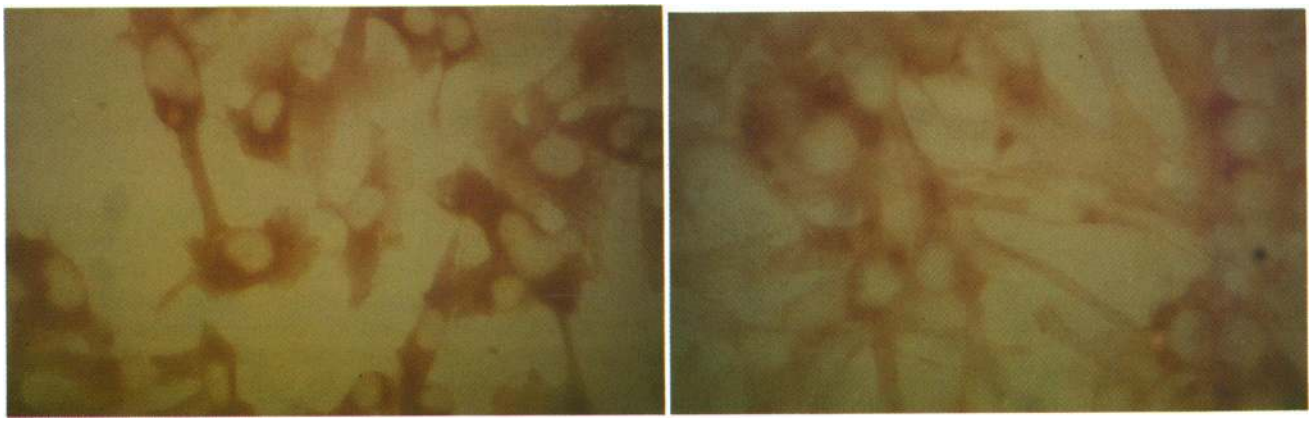

**Figure 9.** Immunoperoxidase detection of VEGF in methanol-fixed human glioma cell lines. Staining was more pronounced in the U-251MG cell line (left panel) than in the CH-235MG glioma cell line (right panel). Note the distinctive nuclear outline generated by the diffuse cytoplasmic staining pattern.

and basic forms of FGF (heparin binding growth factor [HBGF-1 and HBGF-2]), putative angiogenic growth factors in GBM (Zagzag *et al.*, 1990), that have no signal sequence for secretion and are immunochemically localized to glioma cell nuclei and cytoplasm. As a further point of distinction, Connolly (1991) notes that VEGF is a more potent angiogenic growth factor than HBGF on a molar basis. Additionally, although HBGF and TGF-$\beta$ act on numerous cell types, it appears that VEGF mitogenic activity is restricted to endothelial cells. Furthermore, HBGF and TGF-$\beta$ induced angiogenesis is accompanied by fibrosis, a histopathological feature conspicuously absent in GBM tumors. Other putative angiogenic substances include angiogenin, which has not been demonstrated to have any direct activities on endothelial cells, and TNF-$\alpha$ and TGF-$\beta$, both of which can actually suppress endothelial cell proliferation. Finally, although VEGF, histamine, and thrombin are all potent vasoactive substances that directly increase $[Ca^{2+}]_i$ in HUVECs, we have never observed a similar $[Ca^{2+}]_i$ increase in HUVECs by HBGF, TGF-$\beta$, EGF, or TNF-$\alpha$ (unpublished observations).

In addition to our conclusive demonstration that VEGF is present in, and secreted from, glioma cells, we report for the first time that constitutive production of VEGF by four human glioma cell lines was enhanced by EGF. This increased VEGF secretion was documented by ELISA, by changes in $[Ca^{2+}]_i$ in HUVECs, and by increased secretion of vWF from HUVECs. We are currently investigating whether the amount and/or persistence of mRNA encoding for various isoforms of VEGF (Tischer *et al.*, 1991) is also regulated by EGF.

Our finding that EGF stimulates VEGF elaboration has important implications in understanding the biology and basic physiology of malignant glial tumors. EGF is a normal constituent of human plasma and may enter

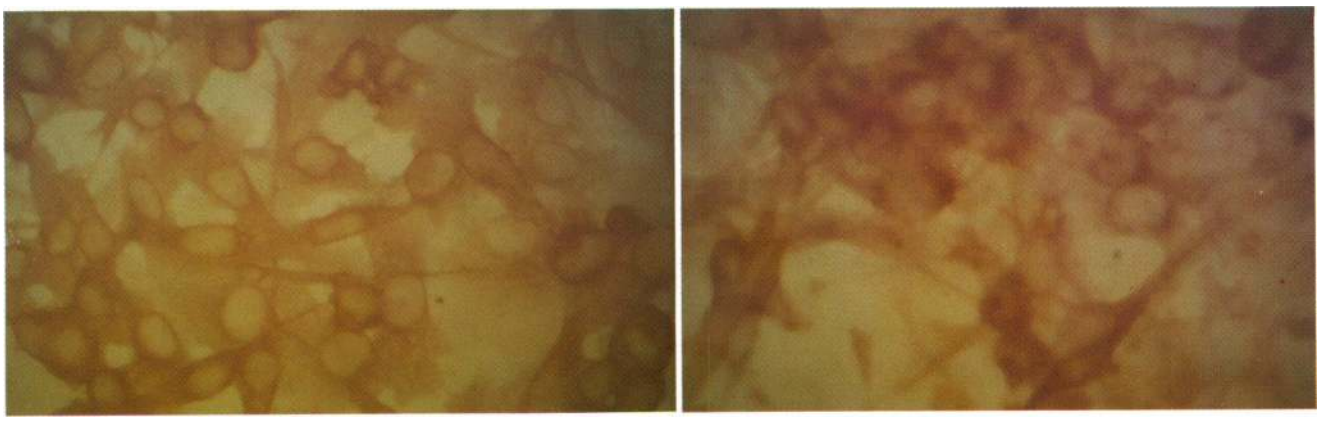

**Figure 10.** Immunoperoxidase detection of EGFr using monoclonal antibody mAb425 methanol-fixed human glioma cell lines. EGFr were present in all glioma cell lines examined; staining patterns for the U-251MG (left panel) and CH-235MG (right panel) glioma cell lines are shown here.

**Table 3.** Summary of detection methods used to demonstrate production of VEGF by five glioma cell lines

| Cell line designation | ELISA | Immunohistochemistry | Calcium release |
|---|---|---|---|
| U-251MG | + | + | + |
| U-105MG | + | + | + |
| D-65MG | + | + | NE[a] |
| D-54MG | + | NE | NE |
| CH-235MG | NE | + | NE |

[a] NE, not examined.

tumor stroma via tumor bed capillaries that have been induced to be "leaky" by VEGF. EGF infiltrating the tumor stroma would be available to bind to glioma cells to increase cellular proliferation and VEGF production. This would represent a positive feedback mechanism and provides a highly plausible explanation for uncontrolled tumor growth, angiogenesis, and other pathological sequlae present in brain tumors and perhaps other malignant cancers (Figure 11). Our paradigm predicts that plasma EGF would be maintained within the intravascular space and would not enter interstitial spaces unless vascular permeability is induced by bioactive compounds (e.g., VEGF) or vessel trauma. Once in the extravascular space, EGF would become biologically important only to cells expressing EGFr. It is relevant that malignant gliomas have been shown by several groups to express or overexpress EGFr constitutively (Sang *et al.*, 1989; Blumenstock *et al.*, 1991; Maruno *et al.*, 1991).

Some GBM tumors, as well as other malignancies, secrete TGF-$\alpha$, which may further augment tumor growth, angiogenesis, edema, and other vascular sequelae. Thus, TGF-$\alpha$ may enhance the positive feedback loop depicted in Figure 11.

In an attempt to explore this working hypothesis further, we have preliminary data that VEGF is present in the MCF-7 human breast carcinoma cell line that has functional EGF receptors that may increase VEGF production (Goldman and Gillespie, unpublished data). Interestingly, expression of functional EGFr in these cells is under hormonal regulation and may represent a ubiquitous mechanism whereby vascularity and cellular proliferation are coregulated in hormone sensitive tissues, such as breast, endometrium, and ovary. Intensive efforts in our laboratory are currently aimed at identifying intra- and intercellular mechanism(s) regulating VEGF-mediated neovascularity in hormone sensitive tissues.

Peritumoral cerebral edema that is characteristically associated with malignant brain tumors presents an immediate and serious life-threatening complication of this disease process. Dexamethasone, a potent glucocorticoid, is clinically useful in ameliorating symptoms in brain tumor patients of increased intracranial pressure. In our experiments, dexamethasone did not reliably diminish the constituitive secretion of VEGF by glioma cell lines. Ohnishi *et al.* (1991) have shown that the $C_6$ rat glioma cell line expresses a protein factor, possibly

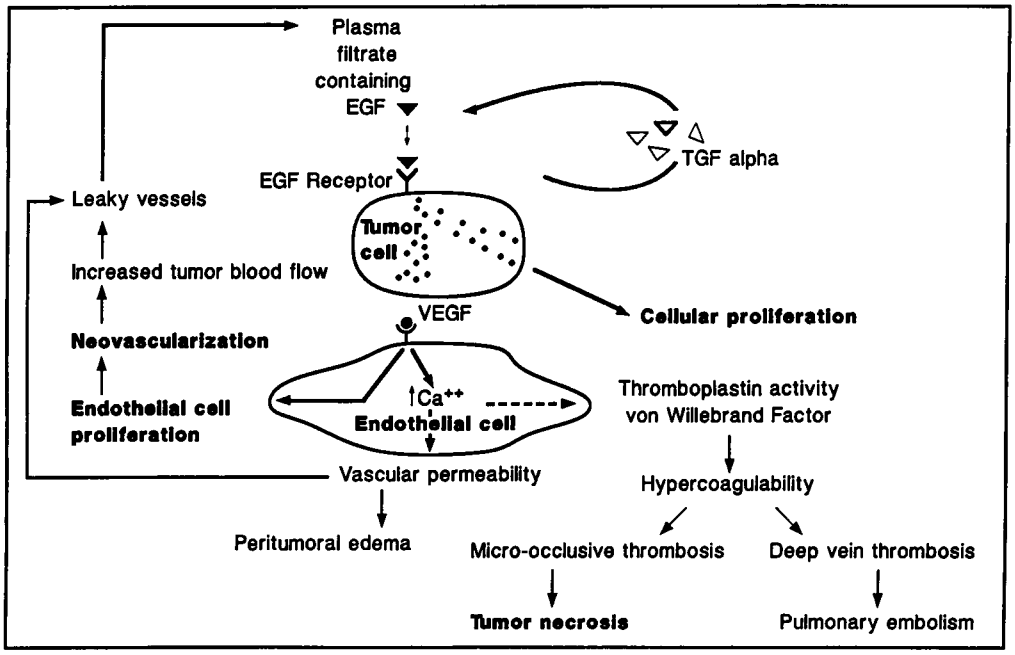


**Figure 11.** A schematic representation of proposed intercellular mechanisms in the development of vascular pathophysiology characteristic of GBM tumors.

VEGF, that stimulated vascular permeability in vivo. The activity of this factor was inhibited by pretreating animals with dexamethasone. They speculate that dexamethasone may act directly on endothelial cells to prevent vascular permeability or indirectly to inhibit glioma cell production of VEGF. Furthermore, Criscuolo *et al.* (1989) have shown that dexamethasone completely blocked human glioma-induced alterations in $[Ca^{2+}]_i$ in HUVECs. Together with our results, these data would suggest that dexamethasone does not reduce cerebral edema by limiting VEGF production but rather serves to antagonize the ability of VEGF to induce vascular permeability related changes in brain capillary endothelial cells.

VEGF-mediated activation of endothelial cells provides a mechanistically simple explanation for many of the pathognomonic histopathological and clinical findings in patients with brain tumors. For example, we have shown that bioactive glioma-derived VEGF produced a marked increase in vWF secretion from endothelial cells and was specifically inhibited by anti-VEGF antibody. In addition to the procoagulant effect of increased endothelial cell vWF secretion, VEGF has been demonstrated to stimulate thromboplastin activity in human endothelial cells (Clauss *et al.*, 1990). Concerted augmentation of platelet aggregation/adherence activity by vWF and enhanced thromboplastin activity (extrinsic cascade) in response to VEGF may lead to increased levels of fibrin split products (Bostrom *et al.*, 1987) and contribute to the increased incidence of thromboembolic phenomena (deep vein thrombosis and pulmonary embolism) seen in brain tumor patients.

As another example, it is a paradox that, despite abundant proliferation of capillaries in GBM, these tumors have variable degrees of necrosis. Although it has been speculated that this necrosis is a result of uncontrolled tumor growth disproportional to blood supply, this explanation does not account for the intense vascularity of these tumors. We contend that the focal necrosis seen in human GBM tumors is due in large part to multiple foci of thrombus resulting from hypercoagulability in the abnormal tumor vessels within the tumor bed. This concept is supported by the work of Clauss *et al.* (1990), who demonstrated that low level TNF-$\alpha$ infusion induces occlusive thrombosis in a VEGF-secreting tumor. Their studies also have shown that VEGF synergizes with TNF-$\alpha$ in stimulating thromboplastin activity in vitro. Furthermore, although TNF-$\alpha$ has inhibitory effects on endothelial cell proliferation, TNF-$\alpha$ in combination with cytokine (Hicks *et al.*, 1989) or FGF (Gospodarowicz, 1989) activation is cytotoxic to endothelial cells. Although it is unknown at present whether VEGF and TNF-$\alpha$ are present in GBM tumors at concentrations cytotoxic to endothelial cells, we recently have demonstrated that human glioma cell lines can produce TNF-$\alpha$ (Bethea *et al.*, 1990, 1992a,b). TNF-$\alpha$ recently has been shown to increase EGFr expression in glioma cells (Adachi *et al.*, 1992), and may further augment the positive feedback of cellular proliferation and angiogenesis in GBM tumors.

The relation between EGFr activation and VEGF secretion in GBM-derived glioma cell lines provides insight into the pathophysiology of GBM tumors and may provide a rational basis for selective interdiction of growth factor-ligand interaction in the treatment of patients with malignant gliomas. Clinical trials are in progress at several institutions including UAB to examine the efficacy of anti-EGFr monoclonal antibodies in the treatment of gliomas (Brady *et al.*, 1991) and other tumor types. Likewise, anti-VEGF antibodies or synthetic EGFr antagonists may be effective adjuncts in treating all of the VEGF-mediated events in the pathogenesis of malignant gliomas; namely, the intense angiogenesis associated with GBM, tumor-associated cerebral edema, and thromboembolic phenomena.

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