Expression and hypoxic regulation of angiopoietins in human astrocytomas[1]

Hao Ding, Luba Roncari, Xiaoli Wu, Nelson Lau, Patrick Shannon, Andras Nagy, and Abhijit Guha[2]

Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada M5G-1X5 (H.D., L.R., X.W., N.L., A.N., A.G.); and Division of Neurosurgery, Division of Neuropathology, Department of Pathology and Laboratory Medicine, The Toronto Hospital and University of Toronto, Ontario, Canada M5T-2S8 (P.S., A.G.)

Vascular endothelial growth factor (VEGF) is a major inducer of tumor angiogenesis and edema in human astrocytomas by its interaction with cognate endothelial-specific receptors (VEGFR1/R2). Tie1 and Tie2/Tek are more recently identified endothelial-specific receptors, with angiopoietins being ligands for the latter. These angiogenic factors and receptors are crucial for the maturation of the vascular system, but their role in tumor angiogenesis, particularly in astrocytomas, is unknown. In this study, we demonstrate that the angiopoietin family member Ang1 is expressed by some of the astrocytoma cell lines. In contrast to VEGF, Ang1 is down regulated by hypoxia. Ang2 was not overexpressed. Expression profiles of low-grade astrocytoma specimens were similar to those of normal brain, with low levels of Ang1, Ang2, and VEGF expression. Glioblastoma multiforme expressed higher levels of Ang1, but not to the same degree as pseudopalisading astrocytoma cells around necrotic and hypoxic zones expressed VEGF, as shown in previous studies. Ang2 expression in the highly proliferative tumor vascular endothelium was also increased, as was phosphorylated Tie2/Tek. The expression profile of these angiogenic factors and their endothelial cell receptors in human glioblastomas multiforme was similar to that in a transgenic mouse model of glioblastoma multiforme. These data suggest that both VEGF and angiopoietins are involved in regulating tumor angiogenesis in human astrocytomas. *Neuro-Oncology 3, 1–10, 2001 (Posted to Neuro-Oncology [serial online], Doc. 00-031, November 7, 2000. URL <neuro-oncology.mc.duke.edu>)*

Received 10 May 2000, accepted 9 August 2000.

[1]This study was supported by an operating grant (Grant No. MT-15688) from the Medical Research Council (MRC) of Canada (A.G.) and a BMS-SQUIBB grant (A.N.). A.G. is supported by a Clinician Scientist Award from the MRC.

[2]Address correspondence and reprint requests to Abhijit Guha, FRCSC, 2-415 McLaughlin Pav., Western Div., UHN, 399 Bathurst St., Toronto, Ontario, Canada M5T-2S8.

[3]Abbreviations used are as follows: GBM glioblastoma multiforme; GFAP, glial fibrillary acidic protein; VEGF, vascular endothelial growth factor.

[4]Ding, H., Roncari, L., Shannon, P., MacMaster, S., Wu, X., Lau, N., Karaskova, J., Gutmann, D., Squire, J., Nagy, A., and Guha, A. (2000) Astrocyte-specific expression of activated Ras results in malignant astrocytoma formation in a transgenic mouse model of human gliomas. Submitted.

The molecular pathogenesis of tumor angiogenesis in any solid tumors has been attributed to "turning on" of the "angiogenic switch," which is modulated by a balance of positive and negative factors released from tumor cells, endothelial cells, and peritumoral inflammatory and immunological cells (Folkman, 1996). Tumor angiogenesis with endothelial hyperproliferation in zones of hypoxia and necrosis is an integral part of the pathological/clinical definition of the malignant astrocytomas termed anaplastic astrocytomas and GBMs[3] (Kleihues et al., 1995).

Among angiogenic modulators, VEGF and angiopoietins are largely specific for regulating angiogenesis. These highly secreted growth factors, in a paracrine fashion, regulate their cognate receptors expressed mainly by the vascular endothelium (Mustonen and Alitalo, 1995). VEGF is highly expressed in human malignant astrocytoma cells and specimens (Feldkamp et al.; Goldman et al., 1993; Plate et al., 1992; Shweiki et al., 1992) and is postulated to promote tumor angiogenesis by activation of cognate VEGF receptors expressed on the endothelium (Plate and Risau, 1995; Plate et al., 1992). This results in the proliferation and migration of endothelial cells, formation of tubules, and generation of immature blood

vessels that lack a mature blood-barrier (Neufeld et al., 1999). Like VEGF receptors, the angiopoietin receptor Tie2/Tek is also an endothelial cell specific tyrosine kinase receptor, though its activation does not lead to endothelial cell proliferation but is speculated to be involved in vessel maturation (Dumont et al., 1993, 1994). Activation of Tie2/Tek leads to recruitment of supportive cells involved in vessel maturation, such as smooth muscle cells, fibroblasts, and pericytes (Dumont et al., 1994), consistent with the observation that Tie2/Tek is activated (phosphorylated) in both the quiescent and actively proliferating vascular endothelium (Wong et al., 1997). The critical involvement of Tie2/Tek in neovascularization is demonstrated by knockout mice, which die early in embryogenesis with gross maturation abnormalities of the vascular system (Dumont et al., 1994). Aberrant activation of Tie2/Tek can also contribute to vascular abnormalities, as exemplified by a spontaneously occurring activating mutation associated with a family predisposed to developing venous malformations, including those in the brain (Vikkula et al., 1996). Our knowledge about the role of Tie2/Tek activation in human tumors is increasing, with expression levels in breast cancers and gliomas correlating to extent of tumor angiogenesis (Peters et al., 1998; Stratmann et al., 1998). In addition, the functional relevance of Tie2/Tek activation in tumor angiogenesis was demonstrated in mouse tumor models, where blocking Tie2/Tek activation with the soluble extracellular domain of Tie2/Tek resulted in decreased tumor angiogenesis and growth (Lin et al., 1998).

Two angiopoietin family members, angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2), have been identified as ligands for Tie2/Tek (Davis et al., 1996; Maisonpierre et al., 1997), whereas Tie1 remains an orphan receptor. Ang1 and Ang2 are both ubiquitously expressed in embryos, with persistent Ang1 expression in adult tissues, though at extremely low levels in brain (Davis et al., 1996; Maisonpierre et al., 1997; Suri et al., 1996). In contrast, Ang2 expression is mainly restricted to actively undergoing angiogenic tissues such as the uterus and ovaries, where it colocalizes with VEGF, but is restricted to endothelial cells (Maisonpierre et al., 1997). Knocking out Ang1 in mice resulted in embryonic lethality with defects in formation of a mature vascular system, although the rudimentary elements of a vascular network are present, reminiscent of the phenotype observed in mice lacking Tie2/Tek (Dumont et al., 1994; Suri et al., 1996), thus suggesting that Ang1 is a ligand for Tie2/Tek. Overexpression of Ang1 can promote vessel sprouting and branch-like endothelial cell formation in vitro (Koblizek et al., 1998) and in vivo (Suri et al., 1998). The hypothesis that Ang2 is a biological antagonist of Ang1 mediated Tie2/Tek activation came initially from transgenic mice models, where Ang2 overexpression led to a phenotype similar to those of Ang1- or Tie2/Tek-deficient embryos (Maisonpierre et al., 1997). Experimental evidence suggests that there is an interplay between VEGF, angiopoietins, and their cognate receptors on endothelial cells in neovascularization. During the active stage of neovascularization, relatively higher levels of VEGF expression by the stromal cells is combined with increased Ang2 expression by the endothelial cells to induce endothelial cell proliferation, migration, and tubule formation. Subsequent increased expression of Ang1 by the stromal cells, combined with lower levels of Ang2 by the endothelial cells, leads to vascular remodeling and maturation and maintenance (Hanahan, 1997).

Although a role for angiopoietins and Tie2/Tek receptor in embryonic vascular development has been well established, the role(s) of these factors in tumor angiogenesis has been relatively unexplored. In this study, we investigated the expression of angiopoietins and Tie2/Tek in human astrocytomas and a transgenic mouse astrocytoma model, a tumor system where much is known about VEGF and VEGFRs (Plate and Risau, 1995; Plate et al., 1992; Shweiki et al., 1992). We demonstrate that there is correlation between angiopoietins and Tie2/Tek expression and grades of human astrocytomas, with highest expression of angiopoietins and tyrosine-phosphorylation of Tie2/Tek in GBMs. Hypoxia, the main physiological stimulant of neoangiogenesis, which is highly relevant in inducing tumor angiogenesis in GBMs, markedly increases VEGF expression but reduces Ang1 mRNA levels in malignant astrocytoma cell lines. Taken together, our expression data also support a yet-to-be-defined role of angiopoietins interacting with VEGF in astrocytoma tumor angiogenesis.

Materials and Methods

Cell Culture

Established human astrocytoma cells U-87 MG and U-138 MG cells were obtained from American Type Culture Collection (Rockville, Md.). U-343 MG and U-343 MG cells, a gift from B. Westermark (Uppsala, Sweden), were grown in Dulbecco's modified Eagle's medium supplemented with 1 mM glutamine, 100 IU/ml, 100 mg/ml streptomycin, and 10% heat-inactivated fetal calf serum (GIBCO/BRL, Gaithersburg, Md.). Ang1 overexpressing astrocytoma cells were obtained by transfecting them with full-length *ANG1* cDNA in the sense orientation under the pCAGG expression vector composed of the cytomegalovirus enhancer with a chicken β-actin promoter. Human fetal astrocytes were purchased from Clonetics (San Diego, Calif.) and cultured in astrocyte basal medium supplemented with 10% horse serum (GIBCO/BRL) according to the manufacturer's instructions. Mouse astrocytes were isolated from the brains of 2-week-old ICR strain mice or from transgenic mice carrying the oncogenic V^{12}*Ras* transgene under the control of the astrocyte-specific GFAP promoter.[4] The isolation of cells was essentially based on previously described methodology (Hertz et al., 1998), with the cells grown in astrocyte basal medium (Clonetics) supplemented with 10% horse serum (GIBCO/BRL).

Hypoxia Treatment

Confluent cultures of human astrocytoma cells grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum were transferred to a hypoxia chamber with palladium catalyst-induced hypoxia (Becton Dickinson, Cockeysville, Md.). Anaerobic conditions were confirmed using BBL™ dry anaerobic indicator strips (Becton Dickinson), as well as by using an oxygen gas sensor, which

read the oxygen content in the chamber to be approximately 0%. Cells were grown under hypoxic/anoxic conditions at 37°C for different time periods as indicated.

RNA Analysis

Total RNA from cultured cells was isolated using the RNeasy mini kit (Qiagen, Valencia, Calif.), whereas total RNA from flash-frozen tumor specimens was extracted using Trazol (GIBCO/BRL). For Northern blots, 10 to 30 μg of total RNA was fractionated on 1% agarose-formaldehyde gels and transferred to Hybond nylon membranes (Amersham Life Science, Uppsala, Sweden). Human *ANG1* and *ANG2* cDNA were generously provided by K. Alitalo, Helsinki, FInland, (494 bp *ANG*1 cDNA coordinates +5 to 499 of coding region; 410 bp *ANG2* cDNA coordinates +6 to 416 of coding region) and were used as probes for Northern blot analysis. The VEGF probe has been described previously (Berse et al., 1992). A glyceraldehyde-3-phosphate dehydrogenase probe served as a control for RNA loading. The DNA probes were radiolabeled with ^{32}Pα-dCTP using a random primer labeling kit (Pharmacia, Uppsala, Sweden). Hybridization was performed in ExpressHyb (Clontech, Palo Alto, Calif.) containing 2×10^6 cpm/ml probe, following the manufacturer's instructions, with the last wash in 0.1 × saline sodium citrate, 0.1% sodium dodecyl sulfate at 50°C for 40 min. The probed blots were exposed to Kodak Blue XB-1 films with intensifying screens at –70°C.

In Situ Hybridization

Eighteen pathologically verified astrocytoma specimens (6 low-grade astrocytomas, World Health Organization grade II; 12 GBMs, World Health Organization grade IV) and normal human brain tissue from the University of Toronto and London Brain Tumor Banks were used. All specimens were fixed in 4% formalin and embedded in paraffin, and 5-μm sections were cut for in situ hybridization and subsequent immunohistochemistry. The 430-bp *ANG2* cDNA was used as the template to synthesize antisense and sense ^{35}S-labeled RNA probes by in vitro transcription with ^{35}S-UTP, in the presence of either of T7 or Sp6 RNA polymerase corresponding to the promoters flanking the insert sequences. To enhance tissue penetration and avoid nonspecific background staining, the RNA probes were alkali-hydrolyzed to an average length of 100 to 200 bases. In situ hybridization was carried out as described previously (Plate et al., 1993), except the hybridization was performed at 47°C overnight with 50 ng/ml (1×10^8 cpm/μg) denatured RNA probes. Sections were then dehydrated, developed for 2 weeks, counterstained with hematoxylin and eosin, and photographed.

Immunohistochemistry

Immunohistochemistry was performed according to standard procedures incorporating the avidin-biotin-peroxidase complex, using 3,3′-diaminobenzidine tetrachloride as a chromophore. Paraffin-embedded astrocytoma sections were pretreated with 0.5% pepsin (Sigma, St. Louis, Miss.) in 0.01 M HCl (for Ang1 immunostaining) or with 90 μg/ml Proteinase-K (GIBCO/BRL) (for Ang2 immunostaining) for 45 min at 37°C. Pretreated slides were blocked with 10% goat or rabbit serum (GIBCO/BRL), and then incubated with primary antibodies overnight at 4°C. Primary antibodies used were goat anti-Ang1 (dilution 1:300 for human specimens, 1:640 for mouse brain sections; Santa Cruz Biotechnology, Santa Cruz, Calif.); goat anti-Ang2 (dilution 1:300 for human specimens, 1:300 for mouse brain sections; Santa Cruz Biotechnology), rabbit anti–von Willebrand Factor (dilution 1:2000, DAKO, Carpinteria, Calif.) and monoclonal anti-Tie2/Tek antibody (dilution 1:400; provided by K. Peters, Duke University Medical Center, Durham, N.C.). After washing with phosphate-buffered saline, sections were incubated with either biotinylated rabbit antigoat or biotinylated goat antirabbit or antimouse (Zymed, San Francisco, Calif.) for 40 min at room temperature. The sections were washed 3 times with phosphate-buffered saline and incubated with avidin-biotin-peroxidase complex (Vector Laboratory, Burlingane, Calif.), according to the manufacturer's protocol. Color was developed through the use of metal 3,3′-diaminobenzidine tetrachloride (Pierce, Rockford, Ill.) for 3 to 5 min at room temperature.

Western Blot

Ang1 protein expression in the conditioned media from a confluent 100-mm tissue culture plate of human astrocytoma cells was determined by Western blot analysis with a polyclonal Ang1 antibody (Santa Cruz Biotechnology). Flash-frozen tumor specimens and normal human brain tissue were lysed in RIPA buffer; 100 μg of protein lysate was immunoprecipitated with 1 μg rabbit anti-Tie2/Tek antibody (Santa Cruz Biotech); and 20 μl protein A-Sepharose was separated by sodium dodecyl sulfate–polyacrylamide gel (8%) electrophoresis, then transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The filter was blocked with 5% bovine serum albumin and incubated with monoclonal antiphosphotyrosine antibody (dilution 1:1000; Upstate Biotechnology, Lake Placid, NY). Protein was detected using the enhanced chemiluminescence system (Amersham Life Science).

Results

Expression of Angiopoietins in Human Malignant Astrocytoma Cell Lines and Specimens

Several established human astrocytoma cell lines originating from anaplastic astrocytomas/GBMs were examined for angiopoietin expression by Northern and Western blot analysis. As shown in Fig. 1A, Ang1 mRNA expression was detected in U-87 MG, U-118 MG, U-373 MG cells, as well as in human fetal astrocytes. Levels of Ang1 expression were similar to VEGF mRNA expression, with neither angiogenic factors detected in the U-343 MG cells (Fig. 1A). Western blot analysis was used to detect the highly secreted Ang1 in the supernatant of

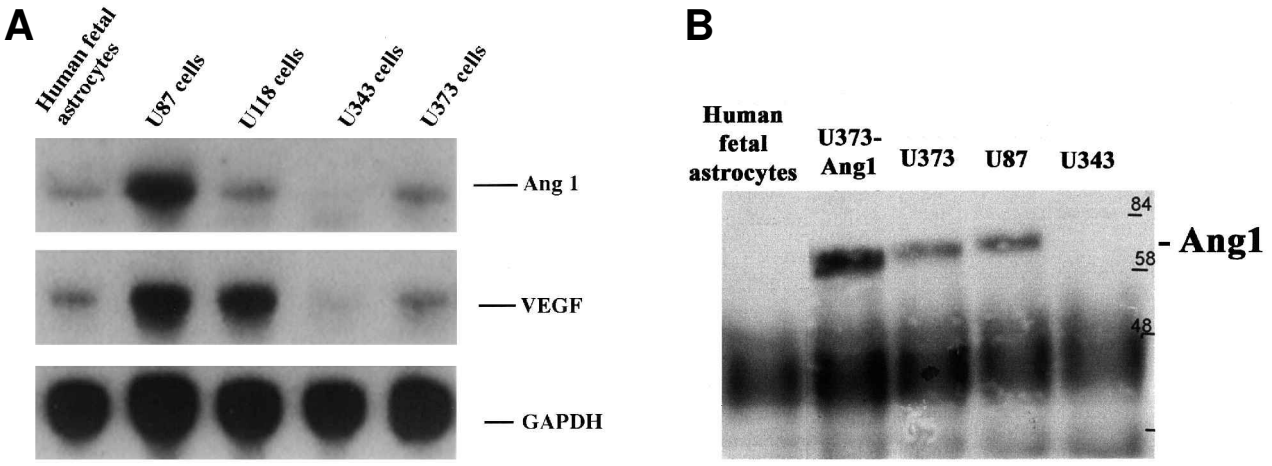

Fig. 1. A. Northern and Western blot analysis of Ang1 mRNA and protein and VEGF mRNA expression in established human astrocytoma cells. Total RNA isolated from human astrocytoma cells (U-87 MG, U-138 MG, U-343 MG and U-373 MG) and human fetal astrocytes as indicated were electrophoresed (30 μg/lane), transferred to membrane, and hybridized to ^{32}P-labeled Ang1 cDNA. The Northern blot was stripped and probed with ^{32}P-labeled VEGF cDNA. GAPDH hybridization is shown as a normalization control. All the tested astrocytoma cells did not express Ang2, as verified by Northern analysis or reverse transcriptase–polymerase chain reaction (RT-PCR). B. Western blot analysis on the supernatant of the astrocytoma cell lines with the anti-Ang1 antibody, the U-373 MG Ang1 cells (transfected to overexpress Ang1) serving as a positive control. All the tested astrocytoma cells did not express Ang2 as verified by Northern analysis or RT-PCR.

the astrocytoma cell lines, with relatively large amounts detected in the U-87 MG and U-373 MG supernatants, but not in the U-343 MG or human fetal astrocytes (Fig. 1B). None of the astrocytoma cells expressed Ang2, a result which was verified with more sensitive reverse transcriptase-polymerase chain reaction analysis (data not shown).

To evaluate angiopoietin mRNA expression levels in human low- and high-grade (GBM) astrocytoma specimens, Northern blot analysis was performed with RNA extracted from flash-frozen samples verified by pathology (Fig. 2). Ang1 or Ang2 mRNA expression was not detected in normal human brain tissue samples, despite adequate levels of mRNA loading as detected by probing with GAPDH. Variable but increased levels of Ang1 and Ang2 mRNA were detected in most GBMs compared with low-grade astrocytomas, which had much lower to undetectable levels (Fig. 2). Adequate amounts of RNA were loaded in all the low-grade astrocytoma lanes, while variable levels were present in the GBMs, most likely due to the presence of tumor heterogeneity and necrosis. The conclusion that Ang1 and Ang2 expression is increased in GBMs versus normal or low-grade astrocytomas is further strengthened if the differences in RNA loading are accounted for by the normalization with the density of GAPDH mRNA (data not shown). In GBMs, 2 species of Ang2 mRNA, of 5 kb and 2.4 kb, were detected that were similar to what has been reported in leukemia cells (Kukk et al., 1997). The Northern blot was stripped and reprobed with VEGF (Fig. 2), demonstrating the previously reported high VEGF mRNA expression in the GBMs compared with low-grade astrocytomas (Plate et al., 1992) (Shweiki et al., 1992).

To determine which cell type(s) in the heterogenous astrocytomas contributes to angiopoietin expression, in situ hybridization and immunohistochemistry were undertaken (Figs. 3 and 4). Sections of normal human brain had weak cytoplasmic, granular staining of Ang1 in neuronal cells and some endothelial cells (Fig. 3A) and had weak staining of Ang2 mainly in endothelial cells (Fig. 4A and 4D), similar to the previous report (Stratmann et al., 1998). In low-grade astrocytoma specimens, Ang1 protein expression was slightly increased in some of the astrocytoma cells (Fig. 3A and 3B), whereas Ang2 expression was confined to endothelial cells (Fig. 4B and 4E). In GBMs, increased expression of Ang1 was detected in most tumor cells (Fig. 3C), although the characteristic increased VEGF expression around necrotic zones in the pseudopalisading astrocytoma cells (Plate et al., 1992; Shweiki et al., 1992) was not detected for Ang1. Another differentiating feature from VEGF expression was that Ang1 was not detected by either immunohistochemistry or in situ hybridization in the GBM-associated tumor vessels. In contrast, the GBM tumor vessels expressed abundant Ang2 (Fig. 4C and 4F).

Induction of Angiopoietin Expression in the Transgenic Mouse Astrocytoma Model

To further determine whether angiopoietin expression was associated with astrocytoma formation, we examined a transgenic astrocytoma mouse model that we developed using GFAP promoter to express oncogenic V^{12}*Ras* specifically in astrocytes.[4] The GFAP-V^{12}*Ras* transgenic mice develop and die from multifocal infiltrative astrocytomas 2 weeks after birth. These GFAP-positive astrocytomas are hypervascular with increased VEGF- and FactorVIII-positive blood vessels compared with normal mouse brain (Fig. 5), similar to malignant human astrocytomas. Immunohistochemistry with anti-Ang1 or Ang2 antibodies demonstrated increased Ang1 expression in the GFAP-positive tumor cells, with Ang2

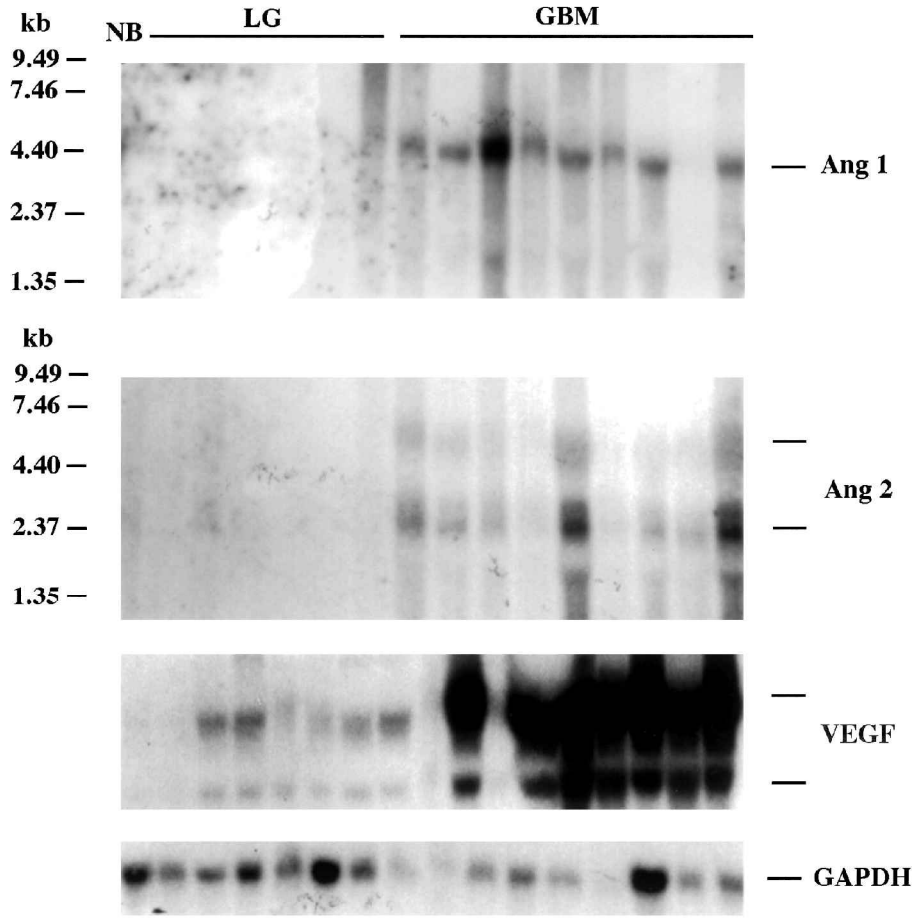

Fig. 2. Northern blot analysis for expression of angiopoietins in flash-frozen surgical human astrocytoma specimens of different grades. The blot containing RNA samples (15 μg/lane) from 1 normal brain (NB), 6 low-grade (LG; WHO grade II), and 9 GBM (WHO grade IV) specimens was hybridized with ^{32}P-labeled Ang1 cDNA probe. The blot was stripped and rehybridized with ^{32}P-labeled human Ang2 and VEGF cDNA. The size of RNA markers is indicated. GAPDH hybridization is shown as a normalization control. Data shown are representative of 3 independent experiments.

detected in the associated tumor vasculature (Fig. 5). Two mouse astrocytoma cell lines derived from these GFAP-V^{12}*Ras* transgenic mice were used to analyze angiopoietin expression by Northern blot analysis. Similar to the human malignant astrocytoma cells, Ang1, but not Ang2 mRNA, was expressed by both the D7 and B8 mouse astrocytoma cell lines (Fig. 6). Both cells also highly expressed VEGF mRNA (Fig. 6).

Down Regulation of Ang1 Expression in Human Astrocytoma Cells by Hypoxia

Hypoxia is one of the main biologically relevant inducers of VEGF and VEGF receptors (Neufeld et al., 1999) and is highly relevant in GBMs characterized by zones of hypoxia and necrosis (Kleihues et al., 1995). Hypoxic up regulation of VEGF has been characterized by our labo-

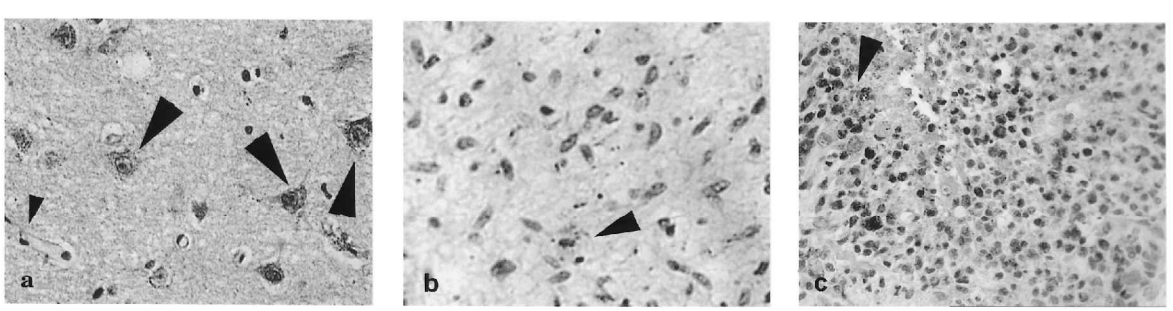

Fig. 3. Immunohistochemical analysis of Ang1 expression in human astrocytoma specimens. A. Normal human brain. Neurons (small arrow) and vascular endothelial cells (large arrows) weakly express Ang1. B. Low-grade astrocytoma. A number of astrocytoma cells (arrow) express higher levels of Ang1 compared with normal brain. C. GBMs. GBM astrocytoma cells (arrows) express abundant Ang1. Original magnification X160.

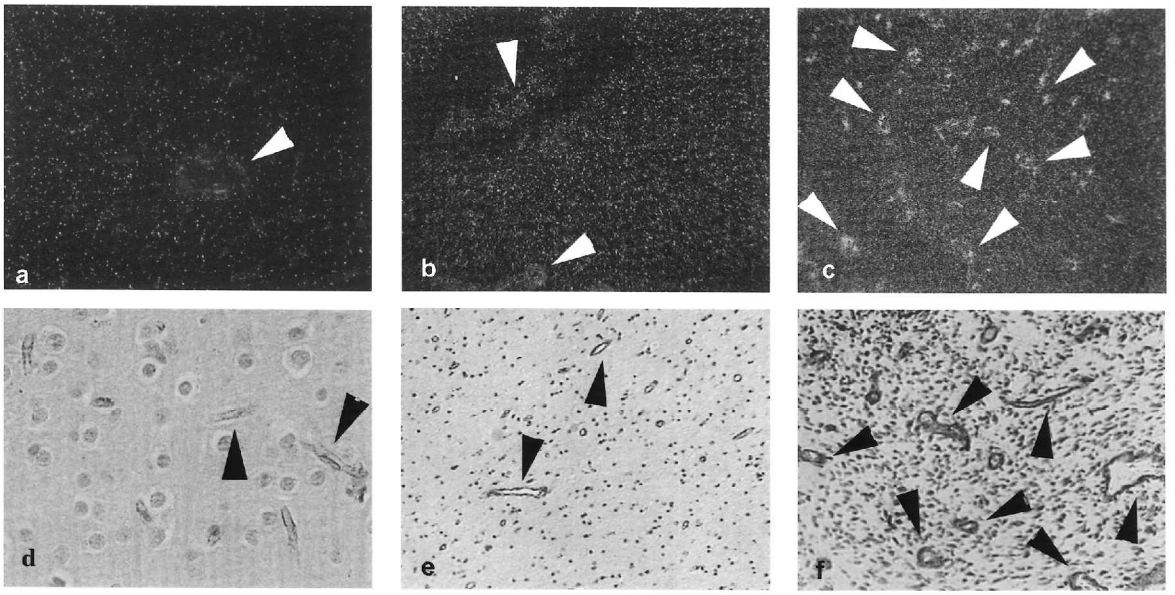

Fig. 4. In situ hybridization (A-C) and immunohistochemical (D-F) analysis of Ang2 expression in human astrocytoma specimens: A and D. Normal human brain. Weak to absent expression of Ang2 in normal brain endothelial cells (arrows); B and E. Low-grade astrocytoma. Increased Ang2 expression in tumor-associated endothelial cells (arrows); C and F. GBMs. Strong Ang2 expression in GBM-associated endothelial cells (arrows). Original magnification A-C, E, and F ×80; D ×160.

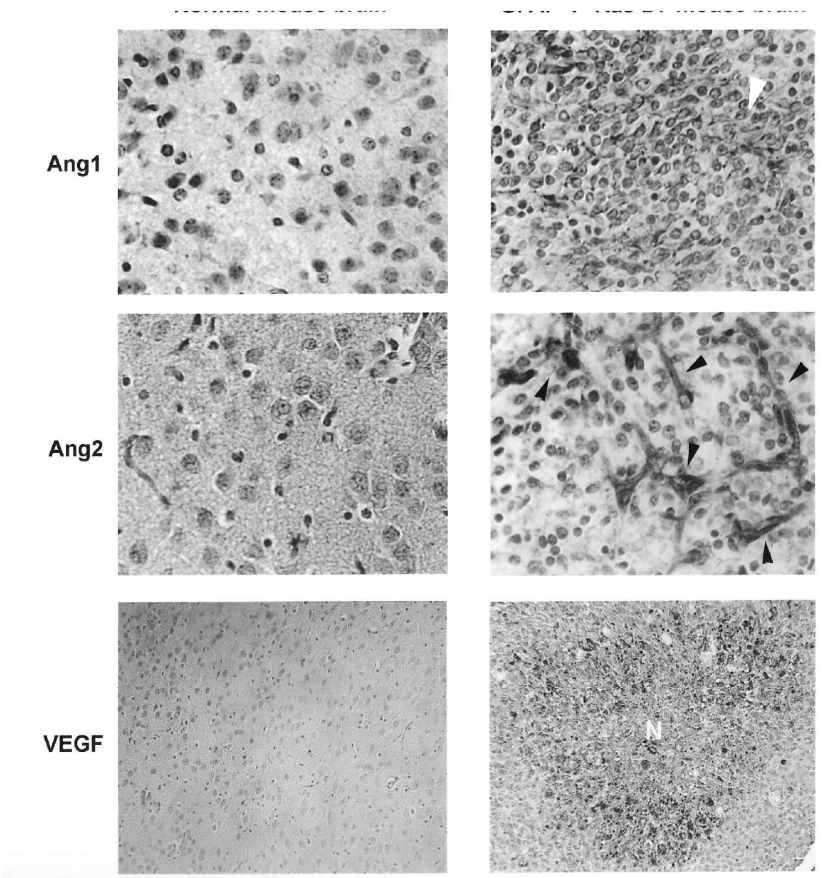

Fig. 5. Induction of angiopoietin expression in a transgenic mouse astrocytoma model developed by overexpressing V^{12}Ras in astrocytes using the human GFAP promoter. The brains from normal ICR strain or 2-week old GFAP-V^{12}Ras chimeras were fixed, and the paraffin sections were used for immunohistochemistry with antibodies against Ang1, Ang2, and VEGF, separately. GFAP-positive mouse astrocytoma cells express Ang1 (white arrow) and mouse astrocytoma–associated tumor vessels show strong Ang2 immunostaining (black arrows). N, necrotic area. Original magnification Ang1 and Ang2 immunostaining ×200; VEGF immunostaining ×80.

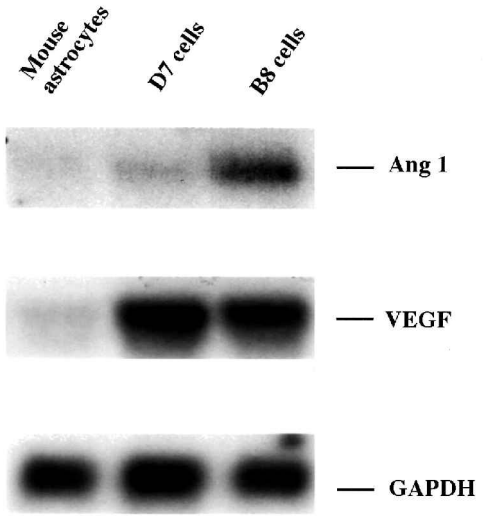

Fig. 6. Expression of Ang1 and VEGF mRNA in tumor-derived cells (D7 and B8 cells) from GFAP-V^{12}Ras transgenic mouse astrocytomas. Northern blot analysis was performed, and total RNA was extracted from normal mouse primary astrocytes, D7 cells, and B8 cells and hybridized with ^{32}P-labeled human Ang1 cDNA or mouse VEGF cDNA probe. The positions of Ang1 and VEGF mRNA are indicated. Expression of GAPDH mRNA was used for normalization control. Neither of the tumor-derived cells expressed Ang2 as determined by Northern blot analysis or reverse transcriptase–polymerase chain reaction (not shown).

ratory in the 4 established human astrocytoma cell lines used in these experiments (Feldkamp et al., 1999). Northern blot analysis demonstrated that, in contrast to the robust VEGF induction in all the cell lines, Ang1 mRNA levels were completely repressed in these cells after 16 h of hypoxia treatment (Fig. 7A). A time course experiment demonstrated that the reduction of Ang1 mRNA in U-87 MG and U-373 MG cells occurred after 8 h or 10 h of hypoxia, respectively. Maximal VEGF hypoxic induction approximated these time points for these cell lines, though increased VEGF mRNA was detected as early as 4 h after initiation of hypoxia (Fig. 7B).

Expression and Activation of Tie2/Tek in Astrocytomas

The expression of Tie2/Tek in varying astrocytoma grades was evaluated by immunohistochemistry with monoclonal anti-Tie2/Tek antibody (Ab33) (Peters et al., 1998). In normal brain specimens, Tie2/Tek was weakly expressed in endothelial cells (Fig. 8A), without any increased Tie2/Tek expression in endothelial cells of low-grade astrocytomas (Fig. 8A). In contrast, significant up regulation of Tie2/Tek expression was present in both the small and large vessels of GBMs (Fig. 8A). Similar to the observation in human breast cancer (Peters et al., 1998), Tie2/Tek expression was increased in areas of neovascularization around the necrotic zones. Immunoprecipitated Tie2/Tek from flash-frozen tissue lysates probed with an antiphosphotyrosine antibody, clearly showed activation of Tie2/Tek in GBMs (Fig. 8B) at levels which were increased 5- to 10-fold compared with normal brain.

Discussion

Human malignant astrocytoma cell lines express Ang1 to variable extent, but not Ang2 because it is expressed mainly by endothelial cells (Maisonpierre et al., 1997; Mandriota and Pepper, 1998). The expression levels of Ang1 mRNA and protein paralleled that of VEGF expression by the astrocytoma cell lines and corresponded to their in vivo tumorigenic potential. For example, the most tumorigenic of the 4 cell lines evaluated was U-87 MG cells (Nister et al., 1991), corresponding to the relative high levels of both Ang1 and VEGF. In comparison, the U-343 MG cells, which do not readily grow in vivo, expressed undetectable levels of both angiogenic factors by Northern or Western blot analysis under normoxic conditions. Expression of angiopoietins and their cognate receptor Tie2/Tek varied between astrocytoma grade and degree of tumor vascularization. Similar to prior observations (Maisonpierre et al., 1997; Mandriota and Pepper, 1998; Wong et al., 1997), normal brain and the associated quiescent vascular endothelium express minimal to nondetectable levels of angiopoietins, with low levels of phosphorylated Tie2/Tek. This expression profile for angiopoietins and Tie2/Tek was not different from low-grade astrocytomas, where, although there is a modest increase in the vascularity, the vessels are still mature with an intact blood-brain barrier (Kleihues et al., 1995).

Comparatively higher levels of Ang1 were detected in all the GBMs, but not to the same level as VEGF expression by these highly malignant and vascularized tumors. In the GBMs, Ang2 was easily detected in endothelial cells, as was Tie2/Tek expression, which was phosphorylated/activated as detected by phosphotyrosine Western blot analysis. This robust expression of VEGF compared with Ang1, with relatively increased Ang2 expression, may promote the formation of the florid but immature tumor vessels associated with GBMs. These GBM-associated blood vessels lack full development of the vessel wall with smooth muscle cells, pericytes, and fibroblasts, as recently reported by an immunohistochemical analysis for these supportive cells (Benjamin et al., 1999). This would lead to formation of immature vessels lacking a mature blood-brain barrier, resulting in peritumoral edema, an improper capillary bed with arteriovenous shunting and intra-tumoral hemorrhage, all characteristic of the GBM tumor vasculature (Kleihues et al., 1995).

Similarities and differences between regulation of VEGF and Ang1 expression by malignant astrocytomas were demonstrated. Activation of the Ras signaling pathway has been demonstrated to be functionally relevant in the proliferation of human malignant astrocytoma cells and GBM specimens (Guha et al., 1997). Based on these observations, we used a GFAP promoter to drive oncogenic-activated V^{12}*Ras* in transgenic mice, specifically in astrocytes. Expression of the transgene results in hypercellular GFAP-positive astrocytic tumors, which in the strong chimeras results in postnatal death by the end of 2 weeks.[4] Compared with wild-type mice brain, these transgenic astrocytomas are similar to sporadic human GBMs, having increased vascularity and overexpressed VEGF, Ang1, and Ang2, with the latter restricted to the endothelium.

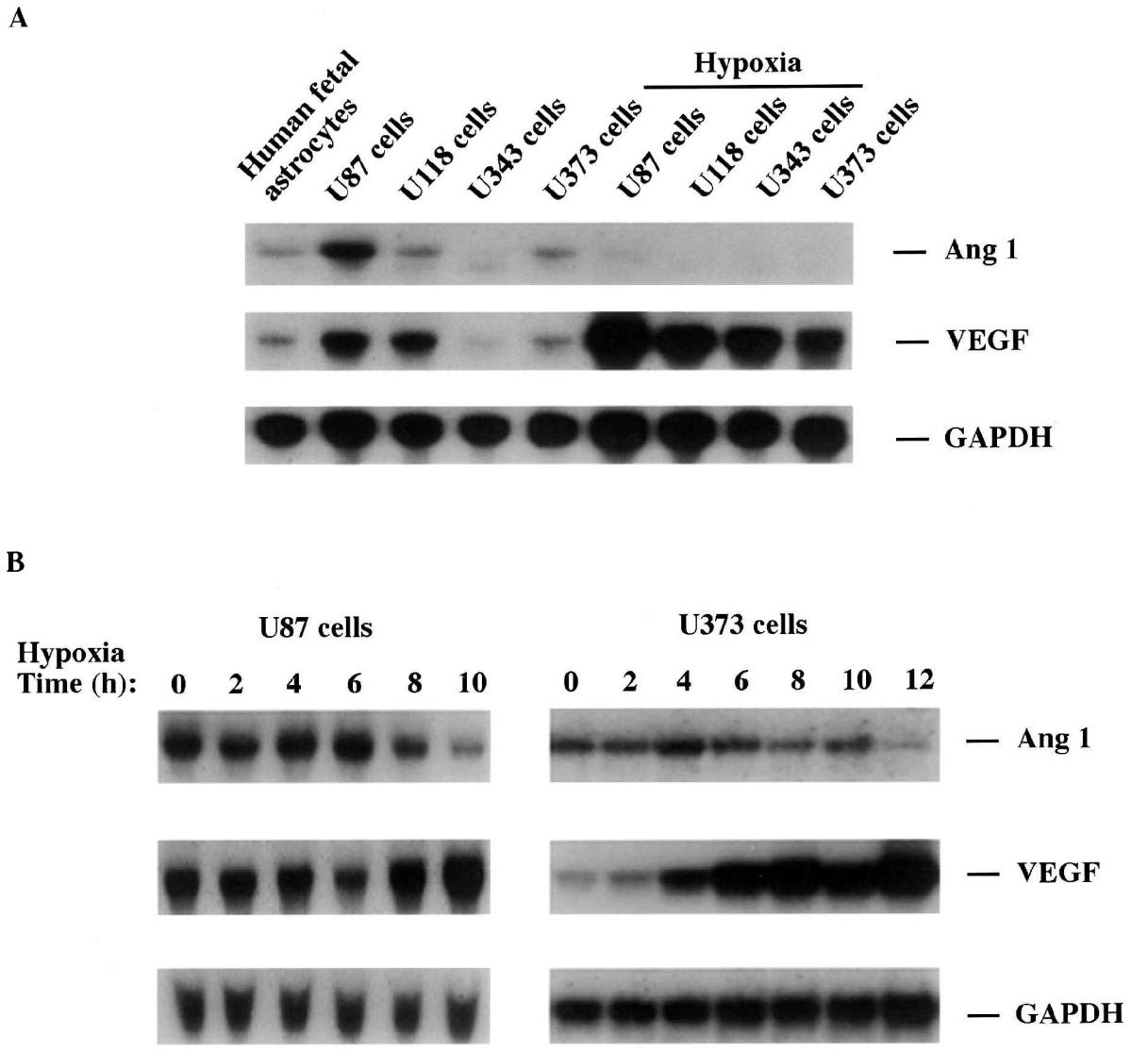

Fig. 7. Decreased Ang1 mRNA expression in human astrocytoma cells under hypoxic conditions. A. Northern blot analysis for Ang1 mRNA expression in human astrocytoma cells under hypoxic conditions. Human astrocytoma cell lines as indicated were exposed to normoxic or hypoxic conditions for 16 h. Total RNA was isolated and analyzed by Northern blot analysis with ^{32}P-labeled Ang1 cDNA or VEGF cDNA probe. Expression of GAPDH mRNA was used for normalization. B. U-87 MG cells and U-373 MG cells were treated with hypoxia for the indicated periods and analyzed as in A. Data shown are representative of 3 independent experiments.

These data serve 2 purposes: first, they help to validate our findings, using a transgenic model, of angiopoietin expression in human GBM specimens. Second, they validate the transgenic astrocytoma model because it shares a molecular-pathologic profile similar to that of human GBMs with respect to tumor angiogenesis.

Activation of Ras, a major mitogenic signaling pathway, also regulates expression of VEGF under normoxic and especially hypoxic conditions, as demonstrated in a variety of human tumor cell lines including astrocytomas (Arbiser et al., 1997; Feldkamp et al., 1999; Okada et al., 1998; Rak et al., 1995). Inhibition of Ras activation by the dominant-negative N^{17}*Ras* mutant or farnesyl-transferase inhibitors in astrocytoma cell lines decreases VEGF expression and prevents its physiologically relevant hypoxic induction (Feldkamp et al., 1999). In contrast, Ang1 mRNA expression was not induced, and in fact was decreased by 8 to 10 h of extreme hypoxia, a time frame within which the predicted VEGF induction was observed. The in vitro data were supported by examining the pseudopalisading astrocytoma cells around zones of necrosis and hypoxia in GBMs. These cells characteristically express the highest levels of VEGF in GBMs due to the hypoxic stress (Plate et al., 1992; Shweiki et al., 1992), but Ang1 expression, although generally increased in GBMs, was not further elevated in these pseudopalisading astrocytoma cells. Whether the decreased Ang1 expression represents active hypoxic repression from putative hypoxia repression elements/factors or is part of the general decreased transcriptional and metabolic cellular machinery noted to occur with hypoxia remains unknown. To

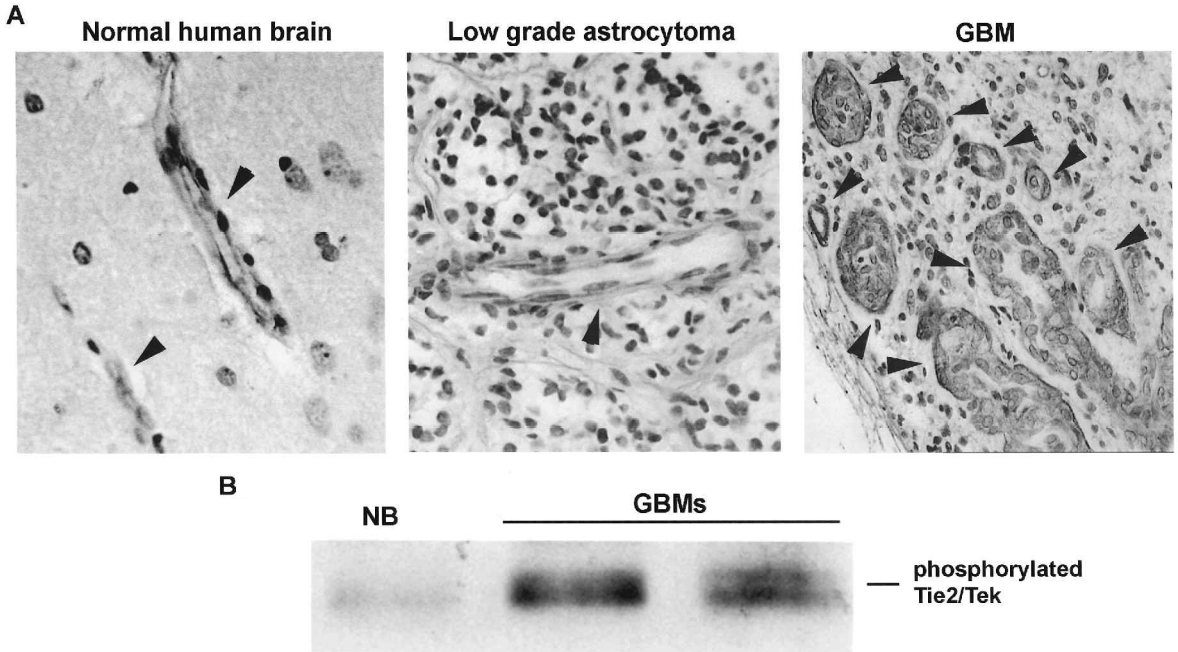

Fig. 8. Expression and activation of Tie2/Tek in human astrocytomas. A. Immunohistochemistry. Sections from normal human brain, low-grade human astrocytoma, and GBM were immunostained with monoclonal anti-Tie2/Tek antibody, and positively stained vascular endothelial cells are indicated by arrows. Original magnification ×200. B. Western blot analysis. Equal amounts of protein from lysates of normal human brain and GBM tissues were extracted and immunoprecipitated with anti-Tie2/Tek antibody and were then probed with antiphosphotyrosine antibody. Tie2/Tek is phosphorylated/activated weakly in normal brain endothelial cells but shows markedly increased levels in GBM-associated endothelial cells.

answer more definitely these questions regarding the mechanisms that lead to decreased hypoxic expression of Ang1, further experiments with the Ang1 promoter would be required. In addition, the functional consequences of angiopoietins and Tie2/Tek activation on the development and maturation of the tumor vascular bed, especially those associated with GBMs, need to be determined. These questions are areas of active research in our laboratories and in other laboratories. It is hoped this knowledge in the future may allow us to exploit potential therapeutic antiangiogenic strategies against these highly vascular and lethal cancers, similar to the strategies being currently employed against VEGF and VEGF receptors.

Acknowledgments

We thank Jennifer Glen and Bodour Salhia, who run the Brain Tumor Bank at the University of Toronto, and the staff at the Brain Tumor Bank at London, Ontario, Canada. The Tumor Bank of Toronto is funded by grants from the Cumming and Lunenfeld Foundation, whereas the bank in London, Ontario, is funded by the National Cancer Institute of Canada and the Brain Tumor Foundation of Canada. We thank Drs. K. Alitalo and K. Peters for providing Ang1 and Ang2 constructs or monoclonal anti-Tie2/Tek antibodies and Nelson Lau and Dr. Gelareh Zadeh for providing technical support in some of the experiments.

References

Arbiser, J.L., Moses, M.A., Fernandez, C.A., Ghiso, N., Cao, Y., Klauber, N., Frank, D., Brownlee, M., Flynn, E., Parangi, S., Byers, H.R., and Folkman, J. (1997) Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 861-866.

Benjamin, L.E., Golijanin, D., Itin, A., Pode, D., and Keshet, E. (1999) Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J. Clin. Invest.* **103**, 159-165.

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

Davis, S., Aldrich, T.H., Jones, P.F., Acheson, A., Compton, D.L., Jain, V., Ryan, T.E., Bruno, J., Radziejewski, C., Maisonpierre, P.C., and Yancopoulos, G.D. (1996) Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* **87**, 1161-1169.

Dumont, D.J., Gradwohl, G.J., Fong, G.H., Auerbach, R., and Breitman, M.L. (1993) The endothelial-specific receptor tyrosine kinase, tek, is a member of a new subfamily of receptors. *Oncogene* **8**, 1293-1301.

Dumont, D.J., Gradwohl, G., Fong, G.H., Puri, M.C., Gertsenstein, M., Auerbach, A., and Breitman, M.L. (1994) Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* **8**, 1897-1909.

Feldkamp, M.M., Lau, N., Rak, J., Kerbel, R.S., and Guha, A. (1999) Normoxic

and hypoxic regulation of vascular endothelial growth factor (VEGF) by astrocytoma cells is mediated by Ras. *Int. J. Cancer* **81**, 118-124.

Folkman, J. (1996) Tumor angiogenesis and tissue factor. *Nat. Med.* **2**, 167-168.

Goldman, C.K., Kim, J., Wong, W.L., King, V., Brock, T., and Gillespie, G.Y. (1993) Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: A model of glioblastoma multiforme pathophysiology. *Mol. Biol. Cell* **4**, 121-133.

Guha, A., Feldkamp, M.M., Lau, N., Boss, G., and Pawson, A. (1997) Proliferation of human malignant astrocytomas is dependent on Ras activation. *Oncogene* **15**, 2755-2765.

Hanahan, D. (1997) Signaling vascular morphogenesis and maintenance. *Science* **277**, 48-50.

Hertz, L., Peng, L., and Lai, J.C. (1998) Functional studies in cultured astrocytes. *Methods* **16**, 293-310.

Kleihues, P., Soylemezoglu, F., Schauble, B., Scheithauer, B.W., and Burger, P.C. (1995) Histopathology, classification, and grading of gliomas. *Glia* **15**, 211-221.

Koblizek, T.I., Weiss, C., Yancopoulos, G.D., Deutsch, U., and Risau, W. (1998) Angiopoietin-1 induces sprouting angiogenesis in vitro. *Curr. Biol.* **8**, 529-532.

Kukk, E., Wartiovaara, U., Gunji, Y., Kaukonen, J., Buhring, H.J., Rappold, I., Matikainen, M.T., Vihko, P., Partanen, J., Palotie, A., Alitalo, K., and Alitalo, R. (1997) Analysis of Tie receptor tyrosine kinase in haemopoietic progenitor and leukaemia cells. *Br. J. Haematol.* **98**, 195-203.

Lin, P., Buxton, J.A., Acheson, A., Radziejewski, C., Maisonpierre, P.C., Yancopoulos, G.D., Channon, K.M., Hale, L.P., Dewhirst, M.W., George, S.E., and Peters, K.G. (1998) Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8829-8834.

Maisonpierre, P.C., Suri, C., Jones, P.F., Bartunkova, S., Wiegand, S.J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T.H., Papadopoulos, N., Daly, T.J., Davis, S., Sato, T.N., and Yancopoulos, G.D. (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* **277**, 55-60.

Mandriota, S.J., and Pepper, M.S. (1998) Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. *Circ. Res.* **83**, 852-859.

Mustonen, T., and Alitalo, K. (1995) Endothelial receptor tyrosine kinases involved in angiogenesis. *J. Cell Biol.* **129**, 895-898.

Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999) Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.* **13**, 9-22.

Nister, M., Claesson-Welsh, L., Eriksson, A., Heldin, C.H., and Westermark, B. (1991) Differential expression of platelet-derived growth factor receptors in human malignant glioma cell lines. *J. Biol. Chem.* **266**, 16755-16763.

Okada, F., Rak, J.W., Croix, B.S., Lieubeau, B., Kaya, M., Roncari, L., Shirasawa, S., Sasazuki, T., and Kerbel, R.S. (1998) Impact of oncogenes in tumor angiogenesis: Mutant K-ras up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 3609-3614.

Peters, K.G., Coogan, A., Berry, D., Marks, J., Iglehart, J.D., Kontos, C.D., Rao, P., Sankar, S., and Trogan, E. (1998) Expression of Tie2/Tek in breast tumour vasculature provides a new marker for evaluation of tumour angiogenesis. *Br. J. Cancer* **77**, 51-56.

Plate, K.H., and Risau, W. (1995) Angiogenesis in malignant gliomas. *Glia* **15**, 339-347.

Plate, K.H., Breier, G., Weich, H.A., and Risau, W. (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature* **359**, 845-848.

Plate, K.H., Breier, G., Millauer, B., Ullrich, A., and Risau, W. (1993) Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. *Cancer Res.* **53**, 5822-5827.

Rak, J., Mitsuhashi, Y., Bayko, L., Filmus, J., Shirasawa, S., Sasazuki, T., and Kerbel, R.S. (1995) Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res.* **55**, 4575-4580.

Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* **359**, 843-845.

Stratmann, A., Risau, W., and Plate, K.H. (1998) Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. *Am. J. Pathol.* **153**, 1459-1466.

Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S., Sato, T.N., and Yancopoulos, G.D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87**, 1171-1180.

Suri, C., McClain, J., Thurston, G., McDonald, D.M., Zhou, H., Oldmixon, E.H., Sato, T.N., and Yancopoulos, G.D. (1998) Increased vascularization in mice overexpressing angiopoietin-1. *Science* **282**, 468-471.

Vikkula, M., Boon, L.M., Carraway, K.L., 3rd, Calvert, J.T., Diamonti, A.J., Goumnerov, B., Pasyk, K.A., Marchuk, D.A., Warman, M.L., Cantley, L.C., Mulliken, J.B., and Olsen, B.R. (1996) Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. *Cell* **87**, 1181-1190.

Wong, A.L., Haroon, Z.A., Werner, S., Dewhirst, M.W., Greenberg, C.S., and Peters, K.G. (1997) Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues. *Circ. Res.* **81**, 567-574.