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Expression of Angiogenic Growth Factor Genes in Primary Human Astrocytomas May Contribute to Their Growth and Progression[1]

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ABSTRACT

Astrocytomas are highly malignant brain tumors and are among the most neovascularized solid tumors. We have investigated the expression of the angiogenic growth factors acidic fibroblast growth factor and transforming growth factor-α, together with its receptor epidermal growth factor receptor, in 30 primary astrocytomas. Both acidic fibroblast growth factor and transforming growth factor-α, together with epidermal growth factor receptor, are found to be greatly overexpressed in these tumors when compared with normal brain. This overexpression of angiogenic growth factors may underlie the intense neovascularization characteristic of astrocytomas.

INTRODUCTION

Astrocytomas are the single most common types of primary intracranial tumors and may be classified according to increasing malignancy as astrocytomas, anaplastic astrocytomas, and glioblastomas. A striking feature of the progression of astrocytomas toward increasing malignancy is the presence of increasing neovascularization (1, 2). The glioblastoma, which represents the most malignant variant of astrocytoma, is perhaps one of the most richly neovascularized solid tumors in terms of vasoproliferation, endothelial cell hyperplasia, and endothelial cell cytology (3).

Angiogenesis, the proliferation of capillary endothelial cells, is a vital component in the development, progression, and metastasis of many human tumors (4, 5). It has been hypothesized that tumors secrete angiogenic peptides which contribute to tumor neovascularization (6) which provides the blood supply necessary for tumor growth (7).

The first angiogenic growth factors to be isolated were aFGF[3] (8–10) and bFGF (11, 12). aFGF and bFGF were found to be structurally related, having a 53% absolute sequence homology (11). The genes encoding for a- and bFGF lack a signal sequence that prevents the secretion of their protein products (10). As with aFGF, the addition of a signal peptide to bFGF enhances its oncogenic potential (13, 14). Whereas aFGF expression is largely confined to neural tissues such as hypothalamus and retina (11, 15, 16), that of bFGF is more ubiquitous, being found in tissues as diverse as brain, kidney, adrenal cortex, bone, macrophages, chondrosarcomas, and hepatomas (11, 15–17). The biological action of aFGF is mediated by a high affinity polypeptide receptor present on the surface of endothelial cells (18–20). Recently, several oncogenes exhibiting 40–50% sequence homology to the FGFs have been identified. They include *int*-2, *hst*/k-*fgf*, and *FGF*-5 (21). Another well-characterized angiogenic growth factor is TGF-α. TGFs are polypeptides that can confer the transformed phenotype to normal cells (22). TGF-α has been isolated from a variety of tumor cells (23), is structurally related to EGF, and binds to the EGF-R encoded by the c-*erb*-B protooncogene (24, 25). TGF-α in addition to being a powerful mitogen has been shown to be a relatively potent angiogenic mediator (26).

The presence of tumor angiogenesis factors has been demonstrated in several glioma cell lines in culture (6, 27, 28). There are no conclusive reports on the expression of these growth factors *in vivo* in primary human astrocytomas. Overexpression of the c-*erb*-B protooncogene which encodes the EGF-R has been reported in 40% of primary glioblastomas examined (29).

In the present study, we report the *in vivo* expression of aFGF, TGF-α, and EGF-R mRNAs in a large population of primary human astrocytomas. mRNA expression in the primary tumors was detected by *in situ* hybridization. Identification of the protein products was established by immunocytochemistry using specific antisera.

MATERIALS AND METHODS

Tissue Collection. For Northern blot analysis tissues were collected intraoperatively and were immediately snap frozen in liquid nitrogen before being stored in a −80°C freezer. For *in situ* hybridization tissue specimens were immersed in ice cold 4% paraformaldehyde and processed as described below. Of the 30 tumors investigated in these studies 23 were obtained from patients with glioblastoma multiforme and 7 from patients with anaplastic astrocytomas. Nonmalignant human brain tissue was obtained during autopsies of 2 persons who died accidentally and intraoperatively from 3 patients who had seizures. The neuropathological classification of the tumors examined in this study is presented in Table 1.

Northern Blot Analysis. Fragments of tissue were immediately placed in ice cold 4 M guanidinium isothiocyanate (Fluka Chemical Co.) before being homogenized by a polytron (setting 6 for 45 s). After being centrifuged for 2 min at 1000 rpm, the supernatant fluid was carefully layered on a cesium chloride cushion and centrifuged (Beckman) in an SW 50.1 rotor at 35,000 rpm, 20°C, for 18 h. Total RNA was then extracted by standard ethanol precipitation after phenol extraction. Aliquots of RNA (25 μg) were heated at 95°C for 2 min in a solution containing 50% formamide, 6% formaldehyde, and running buffer (20 mM 4-morpholinepropanesulfonic acid, pH 7.0, containing 5 mM sodium acetate-1 mM EDTA). The samples were electrophoresed at 35 V overnight on 1% agarose gels containing 6% formaldehyde and running buffer.

The RNA was transferred to Nytran nylon membranes (Schleicher and Schuell), using 10× standard saline citrate transfer buffer and baked at 80°C for 1 h in a vacuum oven. The membranes were then hybridized at 42°C for 16 h with 1×10^6 cpm/ml of random primer-labeled (Amersham) cDNA probe, in a solution containing 50% formamide (Kodak), 0.1% sodium dodecyl sulfate, 5× standard saline citrate,

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[3] The abbreviations used are: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; TGF, transforming growth factor; EGF, epidermal growth factor; EGF-R, EGF receptor; cDNA, complementary DNA; PDGF, platelet-derived growth factor; GFAP, Glial fibrillary acidic protein.

Table 1 *Histopathological classification of the astrocytoma tumors included in Figs. 1-3 with correlation with expression of aFGF, TGF-α, EGF-R, and GFAP genes*

Lane no.	Classification	Grade[a]	Gene expression[b]			
			aFGF	TGF-α	EGF-R	GFAP
1	Glioblastoma	III	++	+	+	+
2	Glioblastoma	III	++	+	+	−
3	Glioblastoma	III	++	+	++	+
4	Glioblastoma	III	++	−	++	+
5	Glioblastoma	III	++	−	++	+
6	Anaplastic astrocytoma	II	++	−	+	+
7	Astrocytoma	I	+	−	+	+
8	Glioblastoma	III	++	−	−	−
9	Anaplastic astrocytoma	II	++	+	+	−
10	Glioblastoma	III	+	+	+++	+
11	Glioblastoma	III	−	−	+	+
12	(Degraded sample)		−	−	−	−
13	Glioblastoma	III	++	+	+	+
14	Glioblastoma	III	+	+	+	+
15	Glioblastoma	III	++	+	+	+
16	Anaplastic astrocytoma	II	++	+	+	+
17	Glioblastoma	III	++	−	−	+
18	Glioblastoma	III	++	+	+	+
19	Anaplastic astrocytoma	II	++	+	+	+
20	Glioblastoma	III	++	+	++	+
21	Glioblastoma	III	++	+	−	+
22	Glioblastoma	III	++	+	++	−
23	Glioblastoma	III	+	++	++	−
24	Glioblastoma	III	++	−	+	−
25	Glioblastoma	III	++	+	+	+
26	Anaplastic astrocytoma	II	+	−	+	+
27	Glioblastoma	III	+	−	+	+
28	Glioblastoma	III	+	−	+	+
29	Anaplastic astrocytoma	II	+	+	+	+
30	Glioblastoma	III	+	+	+	+

[a] Graded according to the World Health Organization standards (40).
[b] +, relative expression; −, no expression.

5× Denhardt's mixture, and 200 μg/ml salmon sperm DNA (Sigma). After washing at 65°C with 0.1× standard saline citrate and 1% sodium dodecyl sulfate, the membranes were subjected to autoradiography at −70°C using intensifier screens. The density of the transcripts was measured by laser densitometry using an AKR densitometer.

The cDNA probes for the present studies include aFGF (10), TGF-α (23), EGF-R (30), β-actin (31), and GFAP (32).

***In Situ* Hybridization.** Fresh tumor tissue was cut into 2-mm thick sections and immersed in ice cold 4% paraformaldehyde for 2–8 h and then allowed to sink in 30% sucrose/phosphate-buffered saline overnight at 4°C to decrease freezing artifacts. Serial cryostat sections (8 μm) of the fixed tissues were subjected to *in situ* hybridization utilizing ^{35}S-labeled cRNA probes as described previously (33).

The specificity of the probes for *in situ* hybridization was controlled by Northern blot analysis and by hybridization of serial sections with control, non-complementary RNA probes.

Immunocytochemistry. Tissues were prepared as described for *in situ* hybridization. The tissue sections were hydrated in phosphate-buffered saline, and endogenous peroxidase activity was suppressed with 0.3% H_2O_2 in methanol and reacted with the appropriate antibody using the Vectastain ABC kit (Vector Laboratories). The tissues were then counterstained with hematoxylin, dehydrated, cleared, and mounted.

The following specific antisera were used in these studies: murine monoclonal antibody to EGF-R. This antibody recognizes a protein epitope on the external domain of the EGF-R distinct from the EGF-binding site (MabG15: 34); sheep polyclonal antibody to TGF-α (Triton BioSciences Inc.); rabbit polyclonal antibody to aFGF (10); rabbit polyclonal antibody to GFAP (Dako Corp.); rabbit polyclonal antibody to factor VIII (Dako Corp.).

RESULTS

Expression of TGF-α and EGF-R mRNAs and of Their Respective Protein Products

Northern Blot Analysis. Northern blot analysis for the expression of TGF-α and EGF-R in primary astrocytomas is shown in Fig. 1. Of the 30 RNA samples examined only one sample was degraded (sample 12). The 4.7-kilobase TGF-α mRNA was expressed in 57% of astrocytomas (17 of 30). The TGF-α panel in Fig. 1 shows weaker expression than in adjacent figures because of sequential hybridization.

EGF-R is encoded by two canonical transcripts of 5.8 and 10.5 kilobases. The predominant 10.5-kilobase transcript is expressed in 73% of astrocytomas (22 of 30), while its 5.8-kilobase transcript is expressed in 40% (12 of 30). Rearrangement of the EGF-R gene gives rise to an additional 3.8-kilobase transcript present in 27% cases (8 of 30). The U1240MG glioblastoma cell line served as a positive control (Fig. 1*C*). Fifty-seven % (17 of 30) of astrocytomas coexpress the TGF-α and EGF-R genes. Normal cerebral cortex did not express the EGF-R gene but expressed the TGF-α gene at low levels and GFAP (Fig. 2).

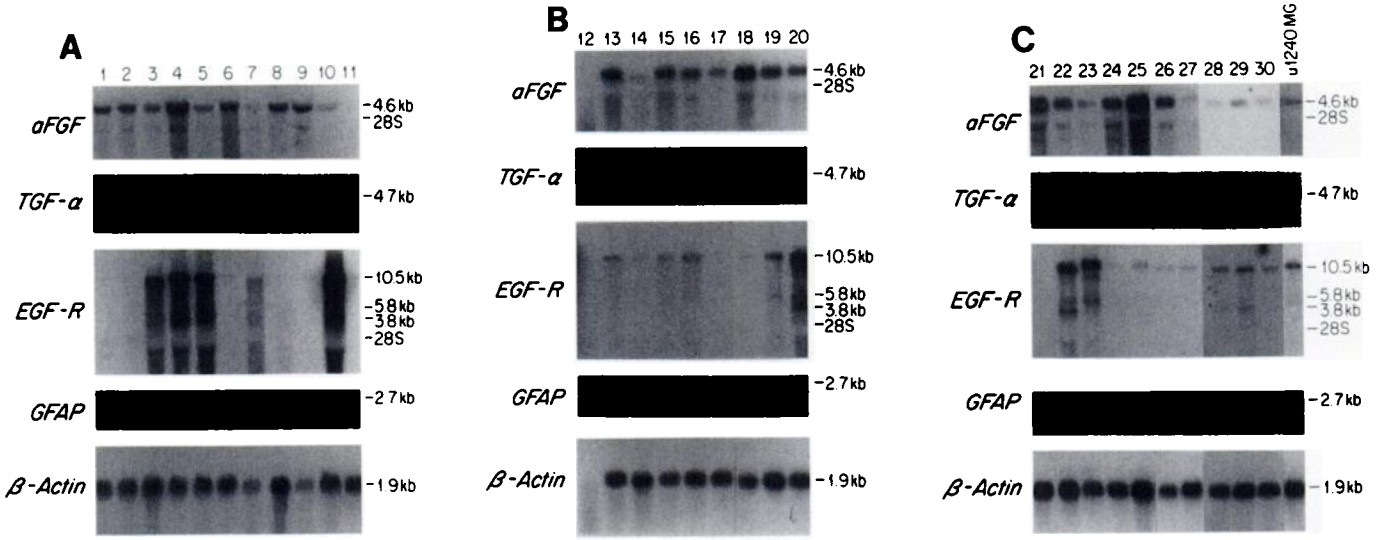

Fig. 1. Northern blot analysis for aFGF, TGF-α, EGF-R, GFAP, and B-actin genes in primary human astrocytomas. Note novel transcripts for EGF-R [3.8 kilobases (kb)] in *lanes 3, 4, 5, 10, 20, 28,* and *29* arising from gene rearrangement. The U1240MG established glioma cell line serves as a positive control. The RNA in *lane 12* is degraded.

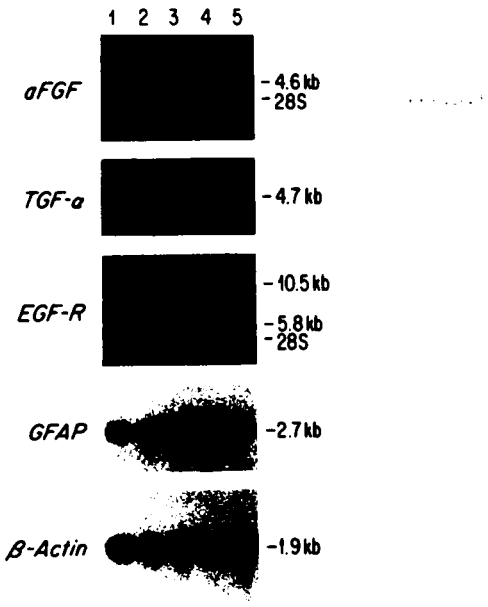

Fig. 2. Northern blot analysis for aFGF, TGF-α, GFAP, and EGF-R in control, nonmalignant brain. kb, kilobase.

***In Situ* Hybridization and Immunocytochemistry for TGF-α and EGF-R mRNAs and Their Respective Protein Products.** Fig. 3 shows a strong expression of EGF-R (*A*) and TGF-α (*B*) mRNAs in the primary astrocytomas. In contrast, there was only a weak expression of TGF-α (*D*) and near background levels of EGF-R (*C*) mRNAs in nonmalignant cerebral cortex. Counterstaining with anti-EGF-R antibody was used in Fig. 3, *B* and *D*. Thus, in Fig. 3*B*, the brown stain identifies glial cells expressing EGF-R-like protein and the superimposed silver grains signify TGF-α mRNA expression. This implies functional coexpression in these cells. Consistent with these data is the expression of their respective protein products. Fig. 4 shows the strong expression of EGF-R-like proteins in a glioblastoma. TGF-α mRNA is expressed at high levels by GFAP-positive glia in a glioblastoma (Fig. 5*A*) and at low levels in normal brain (Fig. 5*C*).

TGFα-like protein was present at very high levels in astrocytoma tissue (Fig. 6*C*) and only at low levels in nonmalignant brain (Fig. 6*A*).

Expression of aFGF mRNA and Its Protein Product

Northern Blot Analysis. The 4.6-kilobase aFGF mRNA transcript is strongly expressed in 93% (28 of 30) of the glioblas-

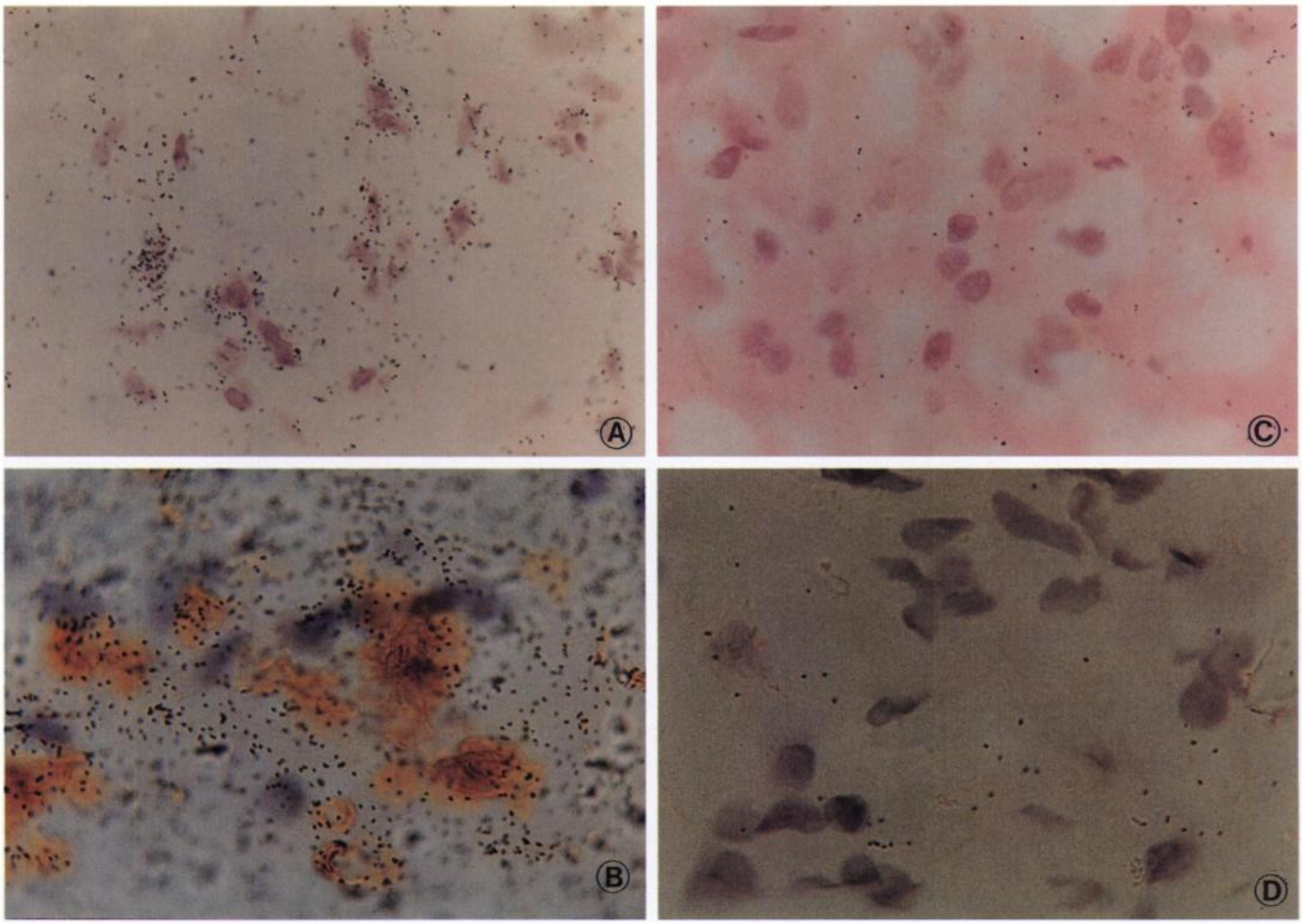

Fig. 3. Localization of EGF-R mRNA in a primary human glioblastoma (*A*) and normal brain (*C*) by *in situ* hybridization and of TGF-α mRNA in cells coexpressing EGF-R-like protein in a glioblastoma (*B*) and in normal brain (*D*). Paraformaldehyde-fixed frozen sections were hybridized with ^{35}S-labeled cRNA probes for EGF-R mRNA (*A* and *C*) and TGF-α mRNA (*B* and *D*). Expression of both EGF-R (*A*) and TGF-α (*B*) mRNAs is seen over clusters of tumor cells. *B*, coexpression of both TGF-α mRNA and EGF-R-like protein (brown stain) by the tumor cells. Normal brain did not express EGF-R mRNA (*C*) or its protein product (*D*). TGF-α mRNA was expressed at low levels in the normal brain (*D*).

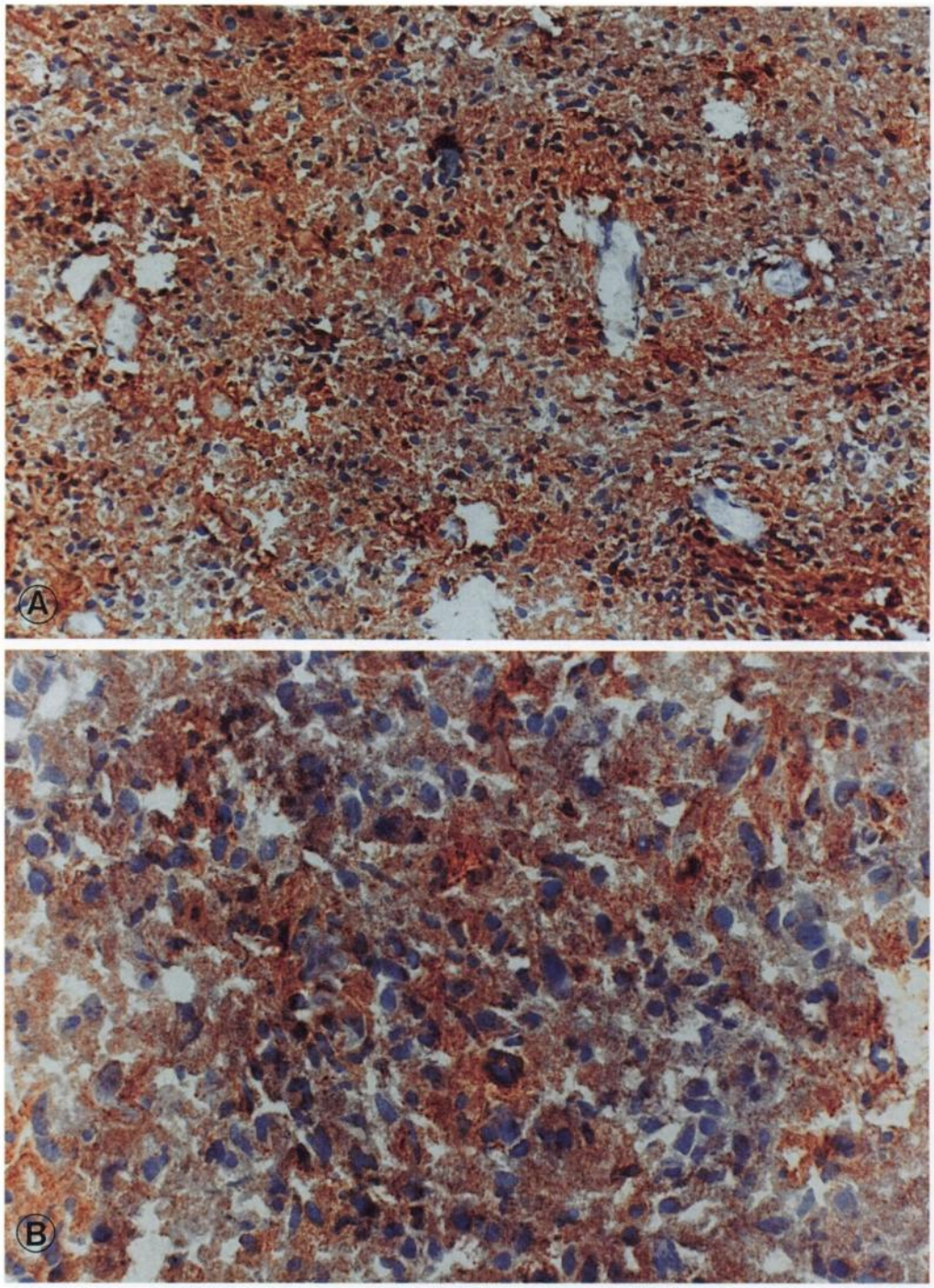

Fig. 4. Immunocytochemistry for EGF-R-like protein in a glioblastoma. Strong staining of EGF-R can be seen in the malignant tissue, using two different magnifications (*A*, × 430; *B*, × 630).

toma samples studied (Fig. 1). Control, nonmalignant cerebral cortex shows a weak expression of the aFGF transcript (Fig. 2). The U1240MG glioblastoma cell line served as a positive control (Fig. 1*C*).

***In Situ* Hybridization and Immunocytochemistry for aFGF mRNA and Its Protein Product.** aFGF mRNA is expressed at high levels in the GFAP-positive tumor cells of a glioblastoma (Fig. 5*B*). In contrast, it is expressed at significantly lower levels in control nonmalignant brain (Fig. 5*D*). Consistent with mRNA overexpression, the presence of a aFGF-like protein is found in abundant amounts in tumors (Fig. 6*D*) and in lower levels in nonmalignant, control brain (Fig. 6*B*).

DISCUSSION

In the present studies we have demonstrated the elevated expression of two angiogenic growth factor genes, aFGF and TGF-α, in a high percentage of primary human astrocytomas. Nonmalignant brain did not express EGF-R mRNA and expressed low levels of TGF-α and aFGF mRNAs. This was accompanied by the lack of expression of EGF-R-like proteins in the nonmalignant brain and by a low level of expression of TGFα-like and aFGF-like proteins. With the exception of tumor #7, a Grade I astrocytoma, all tumor specimens examined in this study were either anaplastic astrocytomas or glio-

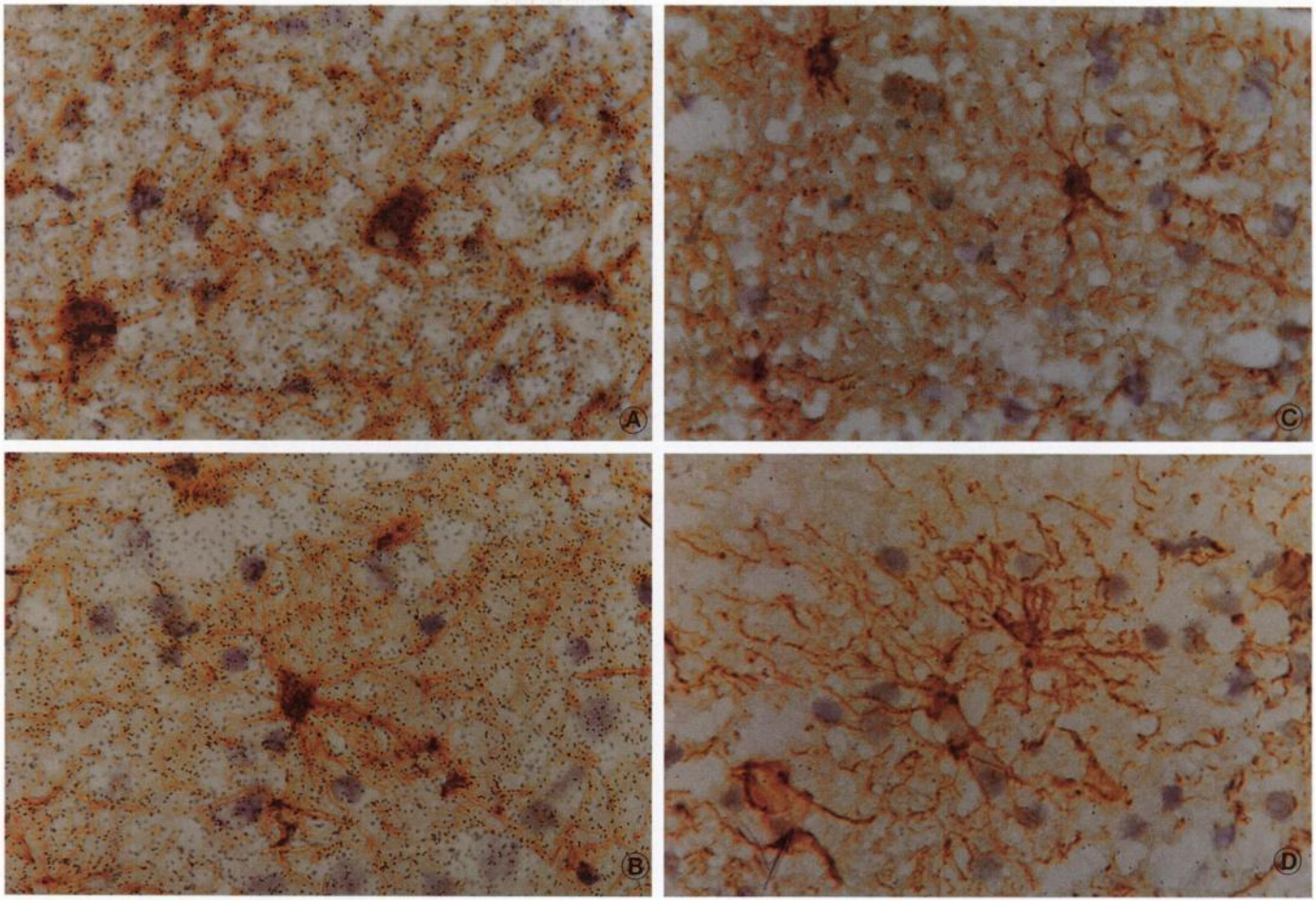

Fig. 5. Localization of TGF-α and aFGF mRNAs in a primary glioblastoma (*A* and *B*) and normal brain (*C* and *D*) by *in situ* hybridization. Paraformaldehyde-fixed frozen sections were counterstained with GFAP and hybridized with ^{35}S-labeled cRNA probes for TGF-α (*A* and *C*) and aFGF (*C* and *D*) mRNAs. Strong expression of both TGF-α (*A*) and aFGF (*B*) mRNAs is seen over clusters of GFAP-positive glial cells of astrocytoma tissue. Normal brain only expresses TGF-α mRNA (*C*) at background and aFGF mRNA (*D*) at low levels.

blastomas and thus represented the malignant end of the spectrum of astrocytic tumors. Increased production of the angiogenic growth factors may underlie the intense neovascularization so characteristic of these tumors. That aberrant expression of the EGF-R gene is involved in the pathogenesis of astrocytomas is suggested by the finding of its consistent amplification and overexpression in approximately 40% of primary human astrocytomas (29, 35). The structural homology between EGF and TGF-α together with their ability to bind to the EGF-R (25) provides the basis for autocrine growth stimulation in the event of coexpression. From our data in primary astrocytomas, coexpression of TGF-α and EGF-R occurred in 57% of the tumors, indicating that this autocrine loop may be instrumental in the neoplastic transformation of glial astrocytes. We have demonstrated the coexpression of TGF-α mRNA and EGF-R-like protein in the same cells. This finding confirms results obtained in established glioma cell lines (28).

While 70% of the gliomas expressed the single 4.7-kilobase TGF-α transcript, 67% of the tumors expressed EGF-R mRNA. Enhanced expression of the 10.5-kilobase EGF-R mRNA was seen in 22 of 30 astrocytomas (73%) (Fig. 1). Normal endothelial cells express high affinity receptors for aFGF (20, 26, 36) and would therefore respond to this peptide. aFGF was originally purified from normal brain (5, 11, 15, 16) and may participate in the induction of angiogenesis during embryogenesis and the maintenance of the blood-brain barrier. In addition to the paracrine stimulation of capillary endothelial cells by aFGF, the mitogenic response by glial cells to aFGF together with expression of its high affinity receptor (36) could also lead to autocrine growth stimulation of these cells (27).

As described above, nonmalignant brain expressed low levels of TGF-α mRNA but not EGF-R mRNA. Thus, the functional role of TGF-α in nonmalignant brain is unknown since there are no detectable amounts of its receptor. In contrast, tumor cells in the primary astrocytomas expressed both TGF-α and EGF-R mRNAs and their respective protein products. This coexpression of a potent mitogen and its receptor suggests the presence of an autocrine mechanism that contributes to the unregulated mitogenic action of TGF-α.

Growth factors may act as either "competence" factors or "progression" factors (37). *In vivo* studies have demonstrated that the synergistic effects of PDGF and TGF-α were required for optimal effect on tissue regeneration (38). The demonstration of coexpression of PDGF and PDGF receptor mRNAs in these astrocytomas (39) along with the coexpression of TGF-α and EGF-R provide a molecular basis for a powerful autocrine loop composed of both competence and progression factors.

Note Added in Proof

Takahashi *et al.* (1990) (41) have reported the expression of acidic and basic FGF, together with TGF-β1 in a wide variety of brain tumors.

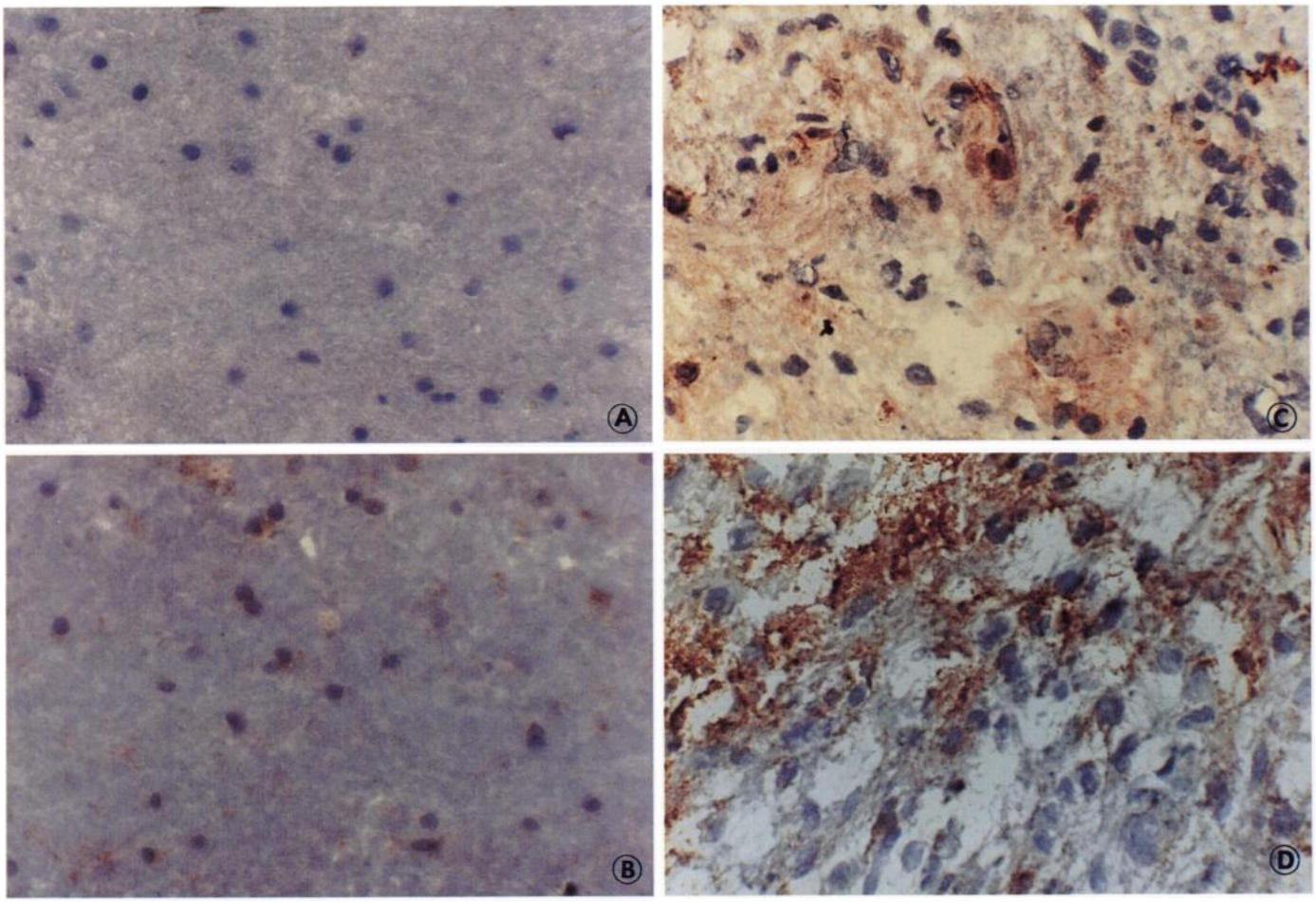

Fig. 6. Immunocytochemistry for TGF-α and aFGF protein in a primary human astrocytoma (*C* and *D*) and nonmalignant brain (*A* and *B*). Strong TGF-α immunostaining is seen in astrocytoma tissue (*C*), while normal cerebral cortex is negative (*A*). Although aFGF staining is present in nonmalignant brain (*B*), far stronger staining is apparent in an astrocytoma (*D*) (× 430).

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