Induction of Vascular Endothelial Tubular Morphogenesis by Human Glioma Cells

A Model System for Tumor Angiogenesis

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Abstract

We have developed two different models of tumor angiogenesis by human brain tumors: one being tube formation by bovine aortic endothelial (BAE) cells cocultured with tumor cells in vitro, and other being in vivo angiogenesis in mice when tumor cells are transplanted into the dorsal sac. We investigated whether tube formation could be induced in BAE cells in type I collagen gel when these cells were cocultured with seven human glioma cell lines. Four of the seven glioma cell lines, which had high levels of basic fibroblast growth factor (bFGF) mRNA, induced tube formation by BAE cells. The tube formation was blocked by coadministration of anti-bFGF antibody. In in vivo model system of tumor angiogenesis in mice, these four cell lines were highly angiogenic. In contrast, with the other three glioma cell lines, which had poor expression of bFGF, BAE cells showed no apparent tube formation. These three cell lines did not efficiently develop capillary networks in mice. The results demonstrated a correlative relationship in the tubulogenesis of BAE cells, bFGF mRNA levels and angiogenesis in mice. The present study with two model systems of tumor angiogenesis suggests that the angiogenesis of some human glioma cell lines is mediated by bFGF, possibly via paracrine control. (*J. Clin. Invest.* 1993. 92:54–61.) Key words: tumor angiogenesis • human glioma cells • tubular morphogenesis • basic fibroblast growth factor • bovine aortic endothelial cells

Introduction

Specific angiogenic molecules released from tumors and macrophages induce angiogenesis (1, 2). The transition from limited to rapid tumor growth is thought to occur along with the transition from the prevascular phase to the vascular phase (3). Angiogenesis is important for tumor growth, but how tumor angiogenesis is controlled remains unclear. Malignantly transformed cells produce various peptide growth factors such as TGF-α, TGF-β, EGF, and basic fibroblast growth factor (bFGF)[1] which control the "autocrine" growth of the malignantly transformed cells (4, 5). Both EGF and TGF-α have been shown to be angiogenic factors (6, 7). In human omental microvascular endothelial cells in culture, EGF or TGF-α has been identified as the most potent growth factor in stimulating cell migration, synthesis of tissue type plasminogen activator (PA), and tube formation in collagen-containing gels (8–13). Other angiogenic factors are acidic FGF and bFGF, which have a high affinity for heparin (14, 15). Proliferation, chemotaxis, and protease production are induced by bFGF in cultured bovine endothelial cells (16–20). In bovine aortic endothelial (BAE) cells, PA synthesis, cell migration, and tube formation are stimulated by bFGF (21), whereas TGF-β antagonizes the effects of bFGF on PA activation and cell migration in these cells (22, 23). A recent study by Kandel et al. (24) has proposed that the expression of bFGF is associated with a switch to the angiogenic phenotype during the development of dermal fibrosarcoma in transgenic mice.

We have been interested in finding which growth factor could be a significant mediator of angiogenesis in human tumor cells. In our present study, we developed two model systems of tumor angiogenesis: first we examined tube formation by BAE-derived endothelial cells cocultured with tumor cell lines in collagen gel in vitro; and, second, we examined angiogenesis in mice in vivo when tumor cells were transplanted into a dorsal air sac. We used seven tumor cell lines derived from human gliomas as tumor cell lines. We propose that angiogenesis during the development of some human gliomas may be controlled by bFGF.

Methods

BAE cell culture. BAE cells were isolated from bovine aortic artery and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 60 μg/ml kanamycin as reported previously (21).

Materials. TGF-α was obtained from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD), TGF-β was from Collaborative Research, Inc. (Lexington, MA), and human recombinant bFGF was obtained from Oncogene Science Inc. (Uniondale, NY). Affinity-purified anti-bFGF antibody (21, 25) and human bFGF cDNA (26) were kindly provided by D. B. Rifkin (New York University Medical Center). Anti-TGF-α antibody was obtained from Oncogene Science Inc. (Uniondale, NY). Human TGF-α (27) and TGF-β (28) cDNAs were donated by R. Derynck (Genentech, Inc., South San Francisco, CA). We obtained [α-^{32}P] dCTP (3,000 Ci/mmol) from Amersham Corp. (Arlington Heights, IL).

Tumor cell lines. Seven glioma cell lines, derived from patients with glioma/astrocytoma, were used in this study: NHG1 and NHG2 were obtained from R. Lipsky, (National Institutes of Health, Bethesda, MD); IN157, IN301, and IN500 were from M. Noble (University of London); U343 from B. Wetermark (Institute of Pathology, Uppsala, Sweden); U251 from American Type Culture Collection, Rockville, MD. These human glioma/astrocytoma cell lines were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 60 μg/ml kanamycin.

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1. *Abbreviations used in this paper:* BAE, bovine aortic endothelial; bFGF, basic fibroblast growth factor; HOME, human omental microvascular endothel; PA, plasminogen activator; t-PA, tissue-type PA; u-PA, urokinase-type PA.

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Northern blot analysis. Northern blot analysis was performed as described previously (9, 25, 29). Endothelial cells were incubated in DMEM containing 10% FBS, and harvested cells were suspended in 4 M guanidinum thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl, and 0.1 M β-mercaptoethanol. We added 2 M sodium acetate (pH 4.0), water saturated phenol, and chloroform successively to the sample. After being mixed vigorously, the sample was left on ice for 20 min and was centrifuged at 10,000 *g* for 20 min. The aqueous phase was aliquoted, mixed with isopropanol, and kept at −20°C for 20 min. The sample was then centrifuged at 10,000 *g* for 20 min to obtain the RNA pellet which was washed with 75% ethanol and dissolved in sterile water. The resulting RNA was fractionated on 1% agarose gel containing 2.2 M formaldehyde and transferred to a Nytran filter (Schleicher & Schuell, Keene, NH). The filter was hybridized to ^{32}P-labeled cDNA probes in Hybrisol (Oncor Inc., Gaithersburg, MD) for 24 h at 40°C, and was then washed at room temperature in 2× SSC and 0.1% SDS, after which it was washed in 0.2× SSC and 0.1% SDS. Autoradiography was carried out with Kodak XAR film.

Tube formation by BAE cells in type I collagen gels and quantitative analysis. 8 vol of type I collagen solution (Nitta Gelatin, Osaka, Japan), 1 vol of 10× DMEM, and 1 vol of 0.05 N NaOH, 200 mM Hepes, and 260 mM $NaHCO_3$ were mixed on ice, poured into 35-mm plastic dishes (Corning Glass Works, Corning, NY), and allowed to form gels at 37°C. BAE cells were plated onto the surface of type I collagen gel in DMEM containing 10% FBS (8). When the BAE cells reached confluence, the medium was replaced with DMEM containing 1% FBS and various growth factors, and the cells were incubated for 3 d. The medium was changed on the second day. On the third day, phase contrast microscopic pictures of each dish were recorded on a still video camera recorder (R5000H; Fuji, Tokyo, Japan), and the total length of tube structures per field was measured, using a cosmozone IS image analyzer (Nikon, Tokyo, Japan), as described previously (11–13). Eight random fields per dish were measured and the total length per field was calculated.

Assays of tube formation in the coculture system. We have established an assay system to determine in tube formation in vessel endothelial cells cocultured with tumor cells in type I collagen gels (11, 12). In this study, we assayed tube formation by BAE cells cocultured with tumor cells. Human tumor cells were cultured in an outer chamber in six-well plate (each well, 38 × 7 mm; Corning Glass Works) in 2 ml DMEM containing 10% FBS as shown in Fig. 1. At confluence, the medium was exchanged for 2 ml DMEM containing 1% FBS. BAE cells were seeded separately in 2 ml DMEM containing 10% FBS, on type I collagen matrigel (1 ml) in culture plates with 0.4-μm filters (Millicell-CM; Millipore Corp., Laboratory Products, Bedford, MA) in an inner chamber (30 × 7 mm) (see Fig. 1). When endothelial cells reached confluence, the serum content of the medium changed from 10% FBS to 1% FBS and the inner chamber was transferred into the outer chamber. In this system, one can expect tubulogenesis to occur in endothelial cells in the collagen gel in the inner chamber when tumor cells cocultured in the outer chamber secrete any angiogenic factor that will pass through the filter of the inner chamber. After 3 d incubation, tube formation in the endothelial cells was quantitated by recording on a floppy video disk. The total tube formation length was analyzed using a cosmozone program (NEC PC-9801; NEC, Tokyo, Japan).

Tumor angiogenesis assay in mice. A dorsal air sac was created in 5- to 7-wk-old male mice according to the method published previously (30–32). Each cell line was suspended in PBS at a concentration of 1 × 10^7 cells/ml, and 0.2 ml of this suspension was injected into a chamber (Millipore Corp.) consisting of a ring (Millipore Corp.) and filter (0.22-μm pore size; Millipore Corp.) on both sides of the ring. This chamber containing BSA (10 mg/ml) or tumor cells was implanted into a dorsal air sac produced in the mouse by injecting 10–15 ml of air. Five mice in each group were killed and carefully skinned on day 5. After the implanted chamber was removed from the subcutaneous air fascia, a ring without filters was placed on the same site and photographed. The area of the air sac fascia with a dense capillary network was quantitated with an image processor (Nexus Inc., Tokyo, Japan) through the photograph.

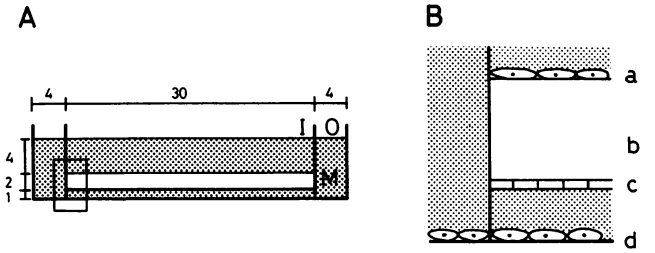

Figure 1. Assay system for tube formation under cocultured system. In this assay system, each well is composed of two chambers: outer chamber (*O*) (38 × 7 mm) and inner chamber (*I*) (30 × 7 mm) (*A*). Tumor cells are grown in medium containing 10% serum in the outer chamber, and at confluence, medium was changed with fresh medium. Separately, HOME cells were seeded on the type I collagen gel (*nonhatched area*) in 2 ml 10% serum containing medium in the inner chamber with 0.4-μm filter (Millipore Corp.) on the bottom. At confluence, the concentration of serum was decreased to 1% in medium (*M; hatched area*) in both chambers, and the inner chamber was transferred into the outer chamber. After incubation for 3 d under the coculture system, the presence or absence of tubular formation of HOME cells in collagen gels was determined. In (*B*), part of the system is magnified: (*a*) endothelial cells on collagen gel; (*b*) type I collagen gel in the inner chamber; (*c*) filter (Millipore Corp.); (*d*) tumor cells in the outer chamber.

Histological analysis of newly synthesized capillary networks was performed. Dorsal skin of mice was fixed in 4% paraformaldehyde in PBS for ~ 20 h. Tissue was then washed in PBS, dehydrated through a series of alcohol, and embedded in paraffin. Tissue sections (5 μm) were melted onto 3-aminopropyltrietho-xysilane–coated slides and stained with hematoxylin and eosin.

Results

Expression of growth factor mRNA in tumor cell lines. To examine whether any angiogenic growth factor was expressed in the brain tumor cell lines, we carried out Northern blot analysis with the cDNAs or oligonucleotides of various angiogenesis-related genes, including aFGF, bFGF, TGF-α, TGF-β, and PDGF-A and -B genes. TGF-β or bFGF gene was found to be expressed in some of the seven glioma cell lines: NHG1, NHG2, IN157, IN301, IN500, U251, and U343. IN301, IN500, U251, and U343 cells expressed higher levels of bFGF mRNAs than NHG1, NHG2, and IN157 cells; there was no apparent expression of bFGF mRNA in IN157 cells (Fig. 2 and Table I). Two different sizes of bFGF transcripts, 7.0 kb and 3.7 kb, were mainly detected in glioma cell lines, and another smaller sized transcript with 1.8 kb was also detected, consistent with a previous report (25). There appeared to be expression of TGF-α mRNA in all the seven cell lines, but their levels were very low except for U251 cells. The levels of TGF-β mRNA with 2.5-kb size were dissimilar in the seven cell lines, and IN301, IN500, and U251 cells showed relatively higher levels of TGF-β mRNA than other cell lines (Fig. 2 and Table I). Our previous study demonstrated that hepatocyte growth factor stimulated cell proliferation and chemotaxis in human microvascular endothelial cells (33); however there was no apparent expression of hepatocyte growth factor mRNA in any of the seven cell lines (unpublished data).

bFGF-induced tube formation in BAE cells. The four human glioma cell lines were found to show high levels of bFGF mRNA, while TGF-α gene was actively expressed only in one cell line, U251. Our previous study has demonstrated that tube formation of human microvascular endothelial cells is effi-

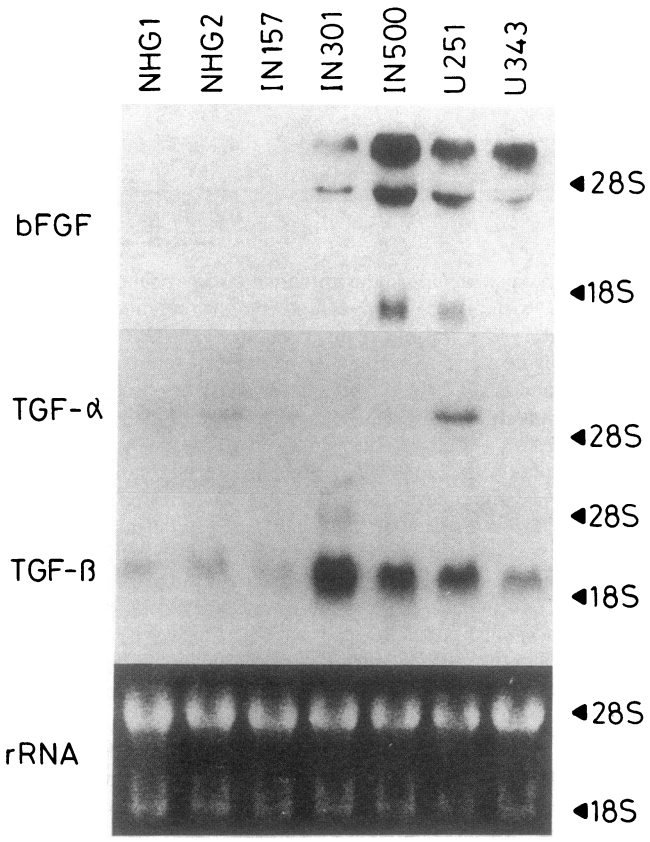

Figure 2. Comparison of cellular levels of bFGF, TGF-α, and TGF-β mRNA in seven glioma cell lines. Total RNAs were extracted from seven cell lines and hybridized with bFGF, TGF-α, and TGF-β cDNA. Autoradiography was carried out with Kodak XAR film after 3-d exposure. Each filter was loaded with 20 μg RNAs. Ribosomal RNA loaded on the gels are shown after staining with ethidium bromide.

ciently observed in the presence of EGF or TGF-α (8, 11, 12). By contrast, BAE cells are highly responsive to angiogenic stimuli by bFGF (21, 34). We therefore investigated whether bFGF or cocultured glioma cells could induce formation of tube-like structures in BAE cells. BAE cells were cultured on a three-dimensional type I collagen gel system and tube formation in these cells was examined when bFGF was present and absent. BAE cells were cultured on the surface of the collagen gel, and after reaching confluence, they had a cobblestone-like appearance in the absence of any exogenous factor (data not shown). In contrast, when the BAE cells were cultured with 10 ng/ml bFGF, branching vessel-like structures appeared inside the gel matrix after 3 d (data not shown). To quantitate the effects of the growth factor on tube formation, the total length of the tube-like structures was determined by image-computer analysis (11, 12). In the absence of any growth factor, tube-like structures were rarely observed in type I collagen gels when BAE cells were assayed (Fig. 3). BAE cells formed numerous tube-like structures in the gels after 3-d incubation with 1 or 10 ng/ml bFGF; these structures were also produced with 100 ng/ml TGF-α at a level comparable with 1 ng/ml bFGF. These findings suggest that tube formation by BAE cells in collagen gel is dependent on bFGF rather than TGF-α. TGF-β has been reported to inhibit cell migration, an angiogenetic activity, in BAE cells (35). Here we examined the effects of this factor on tube formation in BAE cells (Fig. 3). We found that

Table I. Tubulogenesis in BAE Cells, Capillary Network Development in Mice, and Cellular Levels of bFGF and TGF-β mRNA in Glioma Cell Lines

Cell line	Tubulogenesis in BAE*	Angiogenesis in mice‡	Cellular mRNA levels§		Doubling time‖
			bFGF	TGF-β	
					h
NHG1	1.2	1.4	±	+	34
NHG2	1.0	1.0	±	+	30
IN157	1.0	1.3	−	+	44
IN301	3.4	2.7	++	+++	27
IN500	3.5	2.9	+++	++	28
U251	3.1	3.2	++	++	14
U343	2.7	3.5	++	+	47

* Tubulogenesis in BAE cells was determined in a coculture system. Relative tubulogenetic activity is shown, i.e., tubulogenesis normalized with respect to the total length of tube-like structures developed with NHG2 cells, as shown in Fig. 4. ‡ Angiogenesis was determined when capillary networks in the air fascia of mice were developed by tumor cells in a chamber (Millipore Corp.). Each tumor cell line was assayed in four to five mice, as shown in Fig. 6. Relative activity is shown, i.e., the activity when normalized with respect to the networks developed with NHG2. § Cellular levels of bFGF and TGF-β mRNA were determined and were normalized with respect to those appearing in NHG2 cells (Fig. 1). Densitometric analysis was done on Northern blot analysis of three independent experiments. ‖ Cellular doubling times were determined after following growth curves of tumor cell lines in the presence of 10% serum at 37°C.

tube formation by these cells in the presence of bFGF was inhibited by TGF-β of 0.1 and 1 ng/ml. This result agrees well with the results of a previous study by Sato and Rifkin (35).

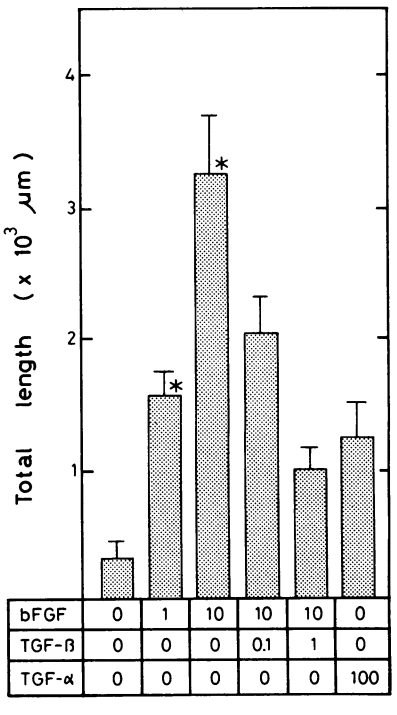

Figure 3. Quantitative analysis of the formation of tube-like structures by BAE cells with bFGF, TGF-β, and TGF-α. When BAE cells plated onto the collagen gel reached near confluence, the medium was replaced with medium containing 1% serum and the indicated doses (ng/ml) of bFGF, TGF-β, and TGF-α. After 3 d of incubation, phase contrast microscopic pictures were recorded with a still video camera recorder, and the total length of the tube-like structures was determined with an image analyzer. Eight random fields per dish were measured and the total length per field was calculated. *Column,* means of three dishes; *bars,* SD; significantly different from the values for control in the absence of any growth factor, $*P < 0.01$.

Model system for angiogenesis of endothelial cells cocultured with tumor cells; effects of specific anti-bFGF antibody. We established a model system for tumor angiogenesis in endothelial cells cocultured with tumor cells, and examined the tumor cell lines to determine which was angiogenic (11). In this assay system, human esophageal cancer cells which produced high level of TGF-α were found to induce tube formation of human microvascular endothelial in type I collagen gel (11). By using this coculture assay system, we determined tube formation by BAE cells when they were cocultured with tumor cells. In this assay system, it was expected that tubulogenesis would be induced in vascular endothelial cell on the surface of the collagen gel in the inner chamber when human tumor cells cultured in the outer chamber secreted any potent angiogenic factor. Of the seven human glioma cell lines, we first characterized three glioma cell lines, NHG2, U251, and IN500.

Fig. 4 shows tubular formation by endothelial cells cocultured with two glioma cell lines, U251 and IN500. BAE cells showed branching vessel-like structures inside the gel after 3 d coculture with U251 or IN500 cells. Cross-sections of the gel demonstrated formation of tube-like structures with lumen inside and junction-like structures with individual cells (Fig. 4, *C* and *D*). Quantitative analysis was done by image computer analysis (Fig. 5). Of the three cocultured cell lines, NHG2 showed no significant tubulogenesis activity with BAE cells, while U251 and IN500 highly induced tubular morphogenesis of BAE cells. About threefold more tube formation was observed with U251 or IN500 than with NHG2 (Fig. 5).

To examine which tumor cell growth factor was responsible for tube formation in the endothelial cells, we tested whether specific anti-bFGF or -TGF-α antibodies inhibited tube formation by BAE cells in the coculture system. Anti-bFGF antibody, but not anti-TGF-α antibody, was found to inhibit tube formation by BAE cells cultured with U251 and IN500 cells (Fig. 4). By contrast, anti-bFGF antibody only slightly inhibited tube formation of BAE cells cocultured with NHG2 cells, while anti-TGF-α antibody did not inhibit this tube formation (Fig. 4). NHG2-induced tube formation in BAE cells appeared to be mediated, in part, by bFGF. Although TGF-α gene was actively expressed in U251 cells (see Fig. 2), anti-TGF-α antibody did not inhibit tubulogenesis in BAE cells, suggesting that TGF-α was not involved in U251-induced formation of tube-like structures in BAE cells. These findings suggested that the U251- and IN500-induced tube formation in BAE cells was mediated by bFGF, possibly through paracrine pathway.

Another model system: angiogenesis of endothelial cells in mice. We used another model of tumor angiogenesis to further examine whether tumor cells were angiogenic. This was an in vivo model in which a ring cavity containing tumor cells was transplanted into a mouse dorsal air sac. Fig. 6 shows photographs of air sac fascia in contact with a chamber containing BSA or tumor cells on day 5 after tumor cell implantation into

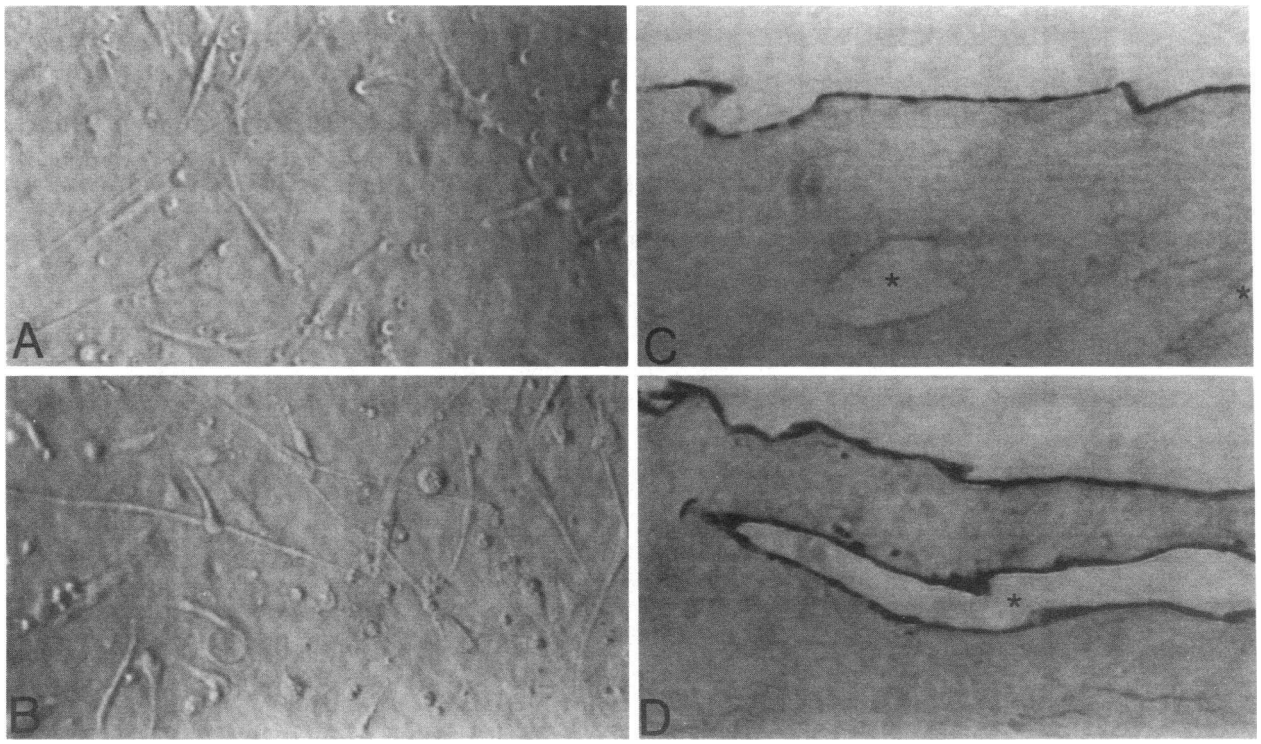

Figure 4. Development of tube-like structures in BAE cells in type I collagen gel, when they were cocultured with glioma cell lines. Development of tube-like structures by BAE cells was examined when BAE cells were cocultured with glioma cells. In this system, each well is composed of two chambers: outer chamber and inner chamber. Human glioma cells were cultured on the outer chamber in 2 ml of medium containing 10% serum. Separately, BAE cells were seeded on the type I collagen gel in 2 ml of medium containing 10% serum in the inner chamber with a 0.4-μm filter (Millipore Corp.) on the bottom. At confluence, the concentration of serum was decreased to 1% and the inner chamber was transferred into the outer chamber. After 3-d incubation in the coculture, the presence or absence of tubular formation of BAE cells in collagen gels was determined. U251 and IN500 cells were cultured in the outer chamber, and BAE cells were plated on type I collagen gel in the inner chamber. On the third day of coculture, microscopic observations were made when BAE cells were cocultured with U251 (*A*) and IN500 (*B*) cells. Numerous tube-like structures were observed. In (*C*) and (*D*), cross-sectional analysis of BAE cells in collagen gel in the coculture with U251 cells showed formation of a lumen (*) inside the tube-like structures. Magnification, 100 (*A* and *B*), and 400 (*C* and *D*).

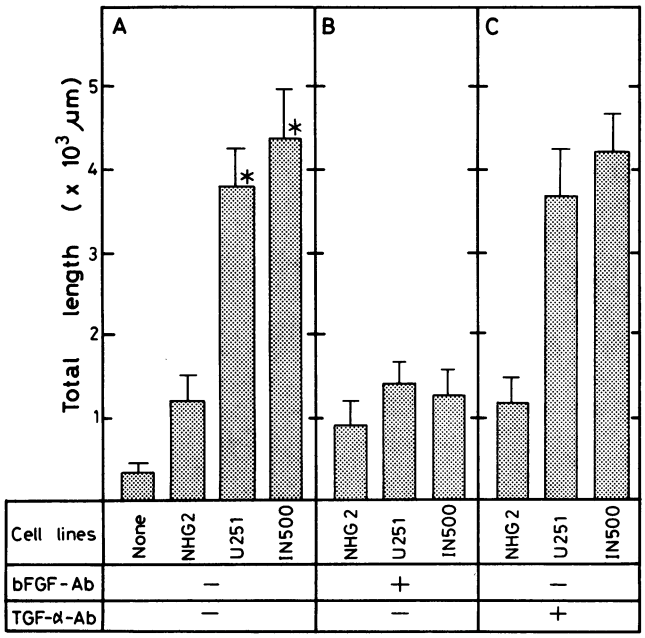

Figure 5. Quantitative analysis of tube formation by BAE cells cocultured with NHG2, U251, and IN500 cells in the absence and presence of anti-bFGF or -TGF-α antibodies. Tublogenesis in BAE cells cocultured with NHG2, U251, and IN50 cells (*A*) as shown in Fig. 3, was examined. In (*B*) and (*C*), anti-bFGF and -TGF-α antibodies, respectively, were present. On the third day of the coculture, quantitative analysis was performed with a still video camera recorder and image analysis as described in the legends to Figs. 3 and 4. *Column,* means of three dishes; *bars,* SD; significantly different from the values for cocultured NHG2 cell, $*P < 0.01$.

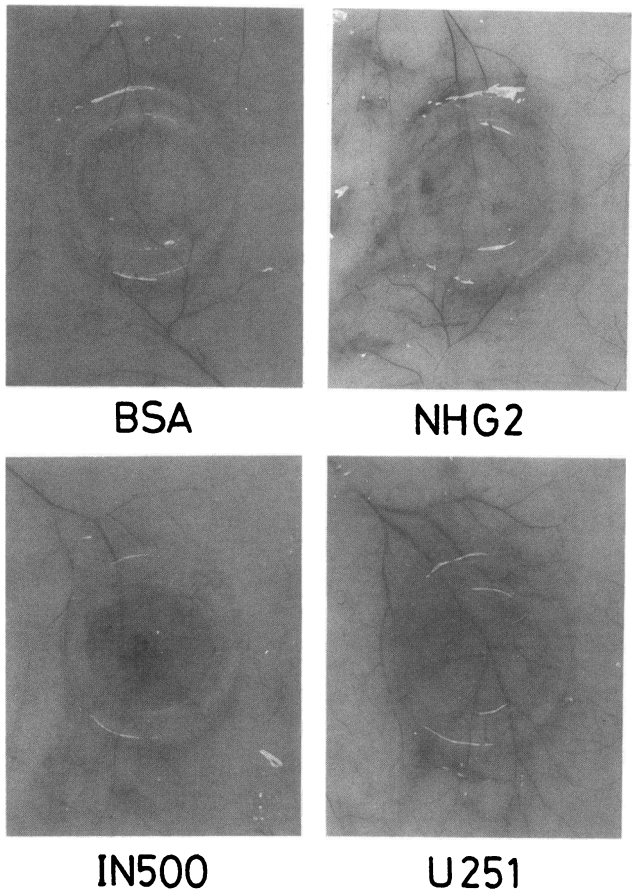

Figure 6. Development of capillary networks in a mouse transplanted with three human glioma cell lines, NHG2, IN500, and U251. Glioma cells at 1×10^7, or bovine serum albumin (*BSA;* 2 mg) were injected into a chamber (Millipore Corp.) with 0.22-μm filters, and the chamber was implanted into a dorsal air sac produced in a mouse by injecting 10–15 ml of air. Mice were killed and carefully skinned on day 5. After the implanted chamber was removed from the subcutaneous air fascia, a ring without filters was placed on the same site and photographed. The size of the chamber ring is 10 mm in diameter.

mice. Of the three tumor cell lines tested, U251 and IN500 were found to induce the development of capillary networks at much greater levels than NHG2. Histological analysis demonstrated new development of capillary networks in the dorsal skin in contact with a implanted chamber containing U251 cells, but not with control chamber containing BSA (Fig. 7). Image-computer analysis of areas of capillary developed in the ring also showed much higher levels of angiogenesis induced by U251 and IN500 cells than by NHG2 cells (Fig. 8). U251 and IN500 were thus found to effectively induce tube formation of BAE cells in the coculture assay and also to effectively induce angiogenesis in mice in vivo.

Tubulogenesis in BAE cells and angiogenesis in mice produced with other glioma cell lines. Of the three glioma cell lines (NHG2, IN500, and U251) used initially, U251 and IN500, which had high levels of bFGF production, induced tubulogenesis in BAE cells in coculture and angiogenesis in mice. We further examined four other glioma cell lines, NHG1, IN157, IN301, and U343. These four cell lines were examined for their ability to induce tubulogenesis in BAE cells in type I collagen gel and for their ability to stimulate development of capillary networks in dorsal air sac fascia in mice. Both of these biological activities of NHG1, IN157, IN301, IN500, U251, and U343 cell lines are listed in Table I when normalized with respect to NHG2 cell activity. Cellular levels of TGF-β and bFGF mRNA, determined by Northern blot analysis (see also Fig. 2) and cell proliferation doubling times are also presented in Table I. TGF-β mRNAs were expressed at various levels in all seven cell lines. The four cell lines (IN301, IN500, U251, and U341), which expressed higher levels of bFGF mRNA, induced higher levels of tubulogenesis in BAE cells in the coculture system and higher levels of neovasculization in mice, though U343 showed relatively lower tubulogenic activity of BAE cells than the other three cell lines (Table I). NHG1, NHG2, and IN157, which did express only a low levels of bFGF, did not show high vasculization activity in either angiogenesis model system.

Discussion

In this study, we developed two models of tumor angiogenesis. In one, we determined tube formation in endothelial cells cocultured with tumor cells in type I collagen gel and in the other, we determined angiogenesis in mice when tumor cells were transplanted into the dorsal air sac. In the former system, we have recently reported that a human esophageal cancer cell line producing TGF-α induces tubulogenesis of endothelial cells derived from human omentum microvessels (11). In this study, we used endothelial cells derived from bovine aortic endothelium and tumor cell lines derived from human glioma. Sato and Rifkin (21) have previously reported that bFGF activates cell migration and PA production in BAE cells. In contrast, our previous studies have demonstrated that EGF or TGF-α, in comparison with other growth factors, most effi-

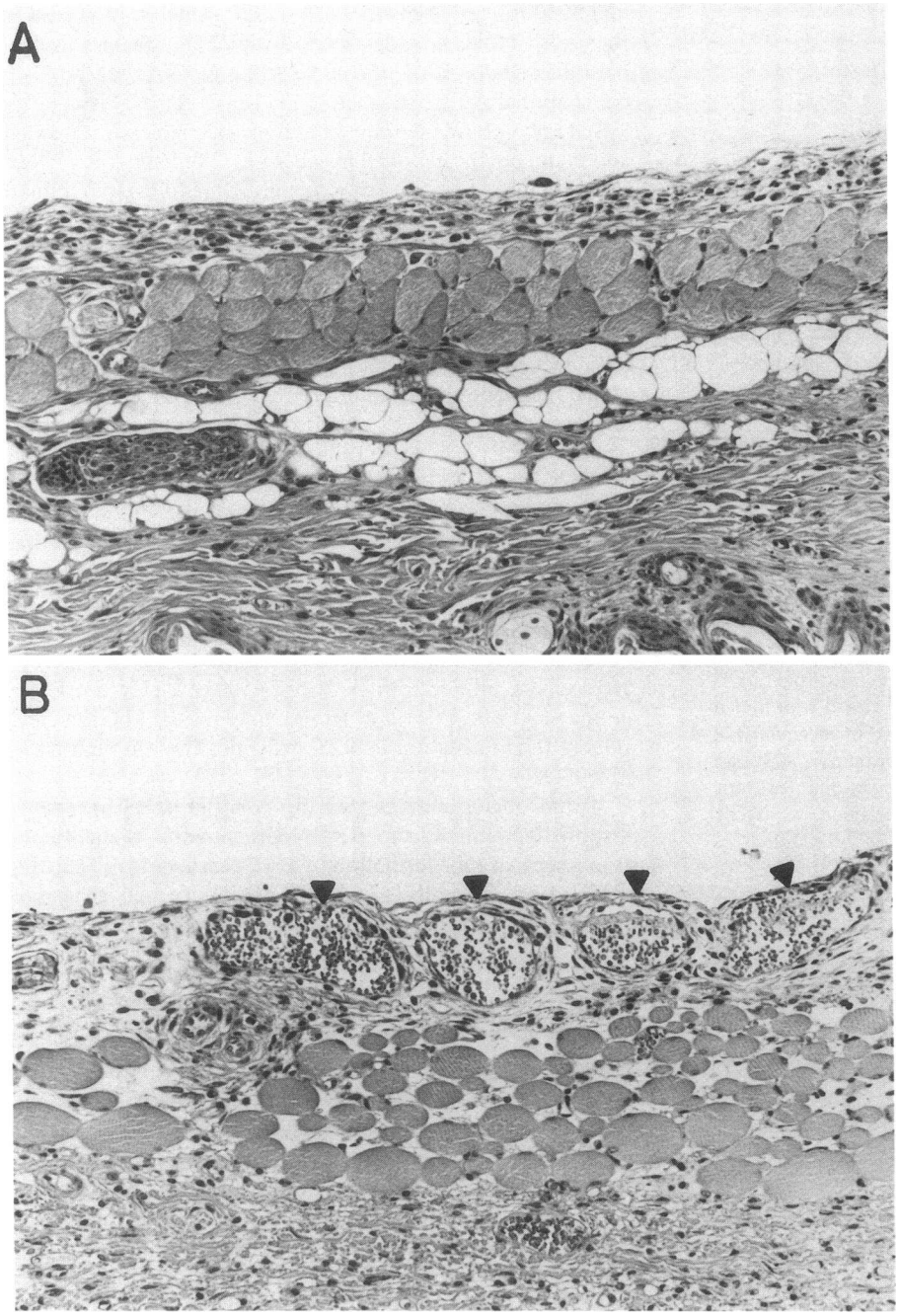

Figure 7. Histological analysis of dorsal skin of mice implanted with glioma cells. Cross-section of the part of the dorsal skin in contact with the implanted chamber containing either BSA (*control*) (*A*) or U251 cells (*B*). Newly capillary networks are developed as indicated by arrows head in (*B*), but not in (*A*). Magnification, 100.

ciently enhanced cell migration, chemotaxis, PA production, and tube formation in type I collagen gel in human microvascular endothelial cell derived from omental tissues (8, 9, 33). Of the seven glioma cell lines, one cell line, U251, showed much higher levels of TGF-α mRNA (Fig. 1). Human omental microvascular endothel (HOME) cells could induce tube-like structures in collagen gel when they were cocultured with U251 cells (unpublished data). In our present study, we found that bFGF induced formation of tube-like structures in BAE cells in type I collagen gel, but that TGF-α showed only weak tubulogenic activity in BAE cells. These findings suggest that tube formation in BAE cells depends rather more closely on bFGF than on TGF-α or EGF. The TGF-β family inhibits the growth of a variety of normal cells, especially these of epithelial origin, in vitro (36, 37). The effects of TGF-β in bovine capillary endothelial cells are the opposite of those of bFGF, i.e., TGF-β, unlike bFGF, does not enhance PA activation and cell migration in these cells (22, 35). Consistent with these findings, TGF-β in this study inhibited tube formation in BAE cells in the presence of bFGF (Fig. 3). All seven human glioma cell lines tested expressed various levels of TGF-β mRNA (Table I). TGF-β produced from glioma cells might be secreted as inactive forms (35) and the inactive TGF-βs could not affect neovasculization activity in the two angiogenesis model systems in this study.

One could argue why there is such a difference between bovine endothelial cells and human microvascular endothelial cells in terms of ability to form tubes in vitro in response to growth factors. HOME cells produce specifically tissue-type PA (t-PA) rather than urokinase-type PA (u-PA) in response to EGF or TGF-α (9, 10), and tubular morphogenesis (13) of HOME cells by EGF or TGF-α. Tubulogenesis of HOME cells in type I collagen gel by EGF or TGF-α is almost completely blocked by anti-t-PA antibody (13) while the tubulogenesis of

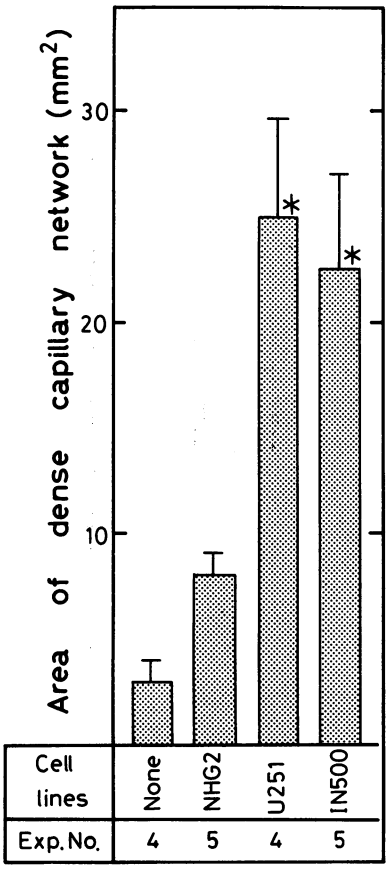

Figure 8. Quantitative analysis of capillary networks developed in mice after transplantation of three glioma cell lines. The area of dense capillary networks was quantitated with an image processor through photographs as shown in Fig. 5. The results are presented as the mean area (mm^2) of the capillary networks inside the ring of the air fascia; each value represents the results obtained with four to five mice. *Column,* mean; *bars,* SD; significantly different from the value for NHG2 cells, $*P < 0.01$.

HOME cells is not induced by exogenous addition of bFGF (11, 12). On the other hand, IGF-1 or hepatocyte growth factor alone has no inducible effect on tube formation, but co-administration with t-PA can induce tubulogenesis of HOME cells (ref. 13 and unpublished data). IGF-1 or hepatocyte growth factor cannot induce expression of t-PA in HOME cells (13, 33). In contrast, in bovine endothelial cells, cell migration, PA synthesis and tube formation are enhanced in response to bFGF (16–21), but not EGF or TGF-α (unpublished data). These results suggest an indispensable role of PAs (t-PA and u-PA) in growth factor–dependent tube formation by HOME or BAE cells. However, BAE cells may specifically induce u-PA in response to bFGF through their bFGF receptor whereas HOME cells may induce t-PA in response to TGF-α or EGF through their EGF receptor. The differential receptor signaling to induce PAs by growth factors might be likely involved in a difference in the ability to induce tubular structures as well as cell migration between bovine endothelial cells and human vascular endothelial cells. Further study is required to understand such specific signaling to induce tubulaer morphogenesis of endothelial cells at molecular levels.

Tubulogenesis appeared to be enhanced in BAE cells cocultured in collagen gel with IN301, IN500, U251, and U343 cells, but not when these cells were cocultured with NHG1, NHG2, and IN157 cells. The four cell lines including U251 and IN500 expressed much higher levels of bFGF mRNA than NHG1, NHG2, and IN157. Coadministration of anti-bFGF antibody blocked U251- and IN500-induced tubular network formation in the coculture system, while the same antibody had negligible inhibitory effect on NHG2-induced tubulogenesis. The cellular action of bFGF is mediated through interaction with specific cell surface receptors, and bFGF receptors have recently been identified in many cell types (38). FGF, which is produced in many cell types, including endothelial cells, are localized in both the cytoplasm and the nucleus (19, 39, 40), but they lack conventional signal sequence in the genes. Basic FGFs are released into culture medium from both mechanically wounded and intact bovine endothelial cells (41). We have recently observed that bFGF is released into culture medium from human microvascular endothelial cells, and also that TNF-α enhances the secretion of these factors (25). TNF inhibits cell migration and tubulogenesis of human microvascular endothelial cells (8, 9). These findings suggest that TNF-induced bFGF is not involved in angiogenesis of human microvascular endothelial cells. In contrast, Sato and Rifkin (21) have previously reported that neutralizing antibodies against bFGF can block the migration of mouse cells and BAE cells, suggesting an autocrine bFGF loop in these cells. Human glioma/astrocytoma cell lines often produce high levels of bFGF (42–45). Neutralizing antibody against bFGF inhibits the growth of human glioma cell lines in vitro as well as in vivo (46), suggesting autocrine growth control of glioma cell lines by secretory bFGF. However, the mechanism underlying angiogenesis in human brain tumors is not understood. In our present study, we found that anti-bFGF antibody inhibited tube formation in BAE cells cocultured with U251 or IN500 cells (Fig. 5). These glioma cell lines, U251 and IN500, may release bFGF, which induces tube formation in BAE cells, possibly through paracrine control.

Tumor angiogenesis assays with seven glioma cell lines demonstrated a common factor involved in both in vivo angiogenesis in mice and tube formation by BAE cells in vitro (Table I). NHG1, NHG2, and IN157 cells did not induce angiogenesis in mice or tube formation by BAE cell, whereas IN157, IN301, IN500, U251, and U343 cells can induce angiogenesis in both systems (Table I). Experiments with these seven human glioma cell lines demonstrated that cellular levels of bFGF appeared to be closely related both to the formation of capillary networks in mice and to tubulogenesis in the coculture system (Table I). These findings suggest that bFGF is positively involved in both in vivo angiogenesis in mice and in tube formation in BAE cells cocultured with human glioma cell lines.

During the development of dermal fibrosarcoma in transgenic mice, fibrosarcoma acquires an angiogenic phenotype at a late stage, due to the extracellular release of bFGF (24), suggesting the involvement of bFGF in neovasculization in mice. Tumor growth is dependent on angiogenesis, but this dependence varies among tumor cell lines. The onset of angiogenesis in tumor cells appearing at very different times during tumor development in different tumor cell lines (47). Angiogenesis depends upon a balance of angiogenic factors and angiogenic inhibitors (1, 48, 49). Our present study suggests that the interaction of bFGF released from the glioma cells with the vascular endothelial cells is sufficient to induce angiogenesis. These two models of tumor angiogenesis should be useful for further studies of the process of tumor angiogenesis.

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