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Dual Pathways to Tubular Morphogenesis of Vascular Endothelial Cells by Human Glioma Cells: Vascular Endothelial Growth Factor/Basic Fibroblast Growth Factor and Interleukin-8

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In this study, we examined whether human glioma cells are angiogenic in a model using human microvascular endothelial cells, and also which factor is responsible for the glioma-dependent angiogenesis. Tubular morphogenesis in type I collagen gel by human microvascular endothelial cells was stimulated in the presence of 10 and 100 ng/ml of vascular endothelial growth factor (VEGF), 10 ng/ml basic fibroblast growth factor (bFGF) and 10 ng/ml of interleukin-8 (IL-8). Tube formation of the microvascular endothelial cells was assayed in the glioma cell lines IN157 and IN301, co-cultured using the double chamber method. IN301 cells had much higher levels of VEGF, bFGF and transforming growth factor-β mRNA than IN157 cells, whereas the two had similar levels of transforming growth factor-α mRNA. By contrast, IN157 cells had much higher levels of IL-8 mRNA than IN301 cells. IN301-dependent tubular morphogenesis was inhibited by anti-VEGF or anti-bFGF antibody, and the inhibition was almost complete when anti-VEGF and anti-bFGF antibodies were present. On the other hand, IN157-dependent tubular morphogenesis was inhibited by anti-IL-8 antibody, but not by anti-VEGF or anti-bFGF antibodies. These findings demonstrated dual paracrine controls of tumor angiogenesis by human glioma cells. One is mediated through VEGF and/or bFGF, and the other, through IL-8.

Key words: VEGF — bFGF — IL-8 — Glioma cell — Tumor angiogenesis model

Tumor angiogenesis supports enlargement of solid tumors and the metastasis of cancer cells.[1-4] Tumor cells as well as normal cells produce various growth factors and cytokines, among which putative angiogenic factors are epidermal growth factor (EGF), transforming growth factor α (TGFα) and β (TGFβ), angiogenin, tumor necrosis factor α (TNF α), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF).[3-6]

Gliomas produce high levels of bFGF, which may mediate the paracrine control of angiogenesis.[7-9] bFGF has no conventional signal sequence,[10] but it acts as an angiogenic factor in chorioallantoic membrane and corneal bioassays *in vivo,*[11] as well as in an *in vitro* angiogenesis model system with cultured bovine vascular endothelial cells.[12,13] Glioma cell lines also produce high levels of a monocyte-macrophage derived cytokine, IL-8.[14-16] IL-8 stimulates chemotaxis of human umbilical vein endothelial cells, and also angiogenesis in the rat cornea.[17]

In contrast, another associated angiogenic factor is VEGF, which has a signal sequence.[18-20] VEGF is also abundantly produced in gliomas, and VEGF might be a potent angiogenic factor in gliomas in animal models,[21-23] and in humans.[24] VEGF is supposed to mediate angiogenic signaling through interaction with its receptors, Flk-1 or Flt-1.[25-27] Millauer *et al.*[28] have reported that glioma growth is inhibited *in vivo* by a dominant-negative Flk-1 mutant. We have recently reported that overexpression of a proto-oncogene, c-*fos*, induces neovascularization of cells implanted in the rat brain.[29] However, the underlying mechanism through which c-*fos* is correlated with angiogenesis in the brain is not known.

In angiogenesis models, the tubular morphogenesis of bovine aortic endothelial cells *in vitro* and development of capillary networks in the dorsal air sac in mice *in vivo* are enhanced by glioma cells that produce bFGF.[30] The formation of tube-like structures of bovine endothelial cells by human glioma cells depends on bFGF secreted from the glioma cells, suggesting that bFGF exerts paracrine control of glioma-dependent angiogenesis.[30] However, it remains to be clarified whether tubular morphogenesis of human vascular endothelial cells can be modulated by culture with human glioma cells, and if so, which factor(s) is involved. We studied how angiogenesis proceeds in model systems of cultured human

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microvascular endothelial cells.[31] Cell migration, chemotaxis, proliferation and tubular morphogenesis in these cells are all stimulated by exogenous EGF, TGFα and heparin-binding EGF-like growth factor (HB-EGF).[32–35] Furthermore, in an angiogenesis model *in vitro*, in which vascular endothelial cells on type I collagen gel in an inner chamber are cultured with other types of cells in an outer chamber, tubular morphogenesis of bovine endothelial cells is enhanced in response to bFGF produced by human glioma cells.[30] On the other hand, that of human microvascular endothelial cells is enhanced in response to TGFα produced by human keratinocytes or human esophagus cancer cells.[32, 33]

Our previous study indicated that four of seven human glioma cell lines, which have higher levels of bFGF, can induce tube formation *in vitro* by bovine aortic endothelial cells.[30] However, that model was heterogenous: tumor cells were derived from a human source and vascular endothelial cells were from bovine aortic endothelium. In the present study, we investigated which angiogenic factor is involved in the human glioma-dependent development of tubular networks by human vascular endothelial cells. We screened seven human glioma cell lines to see whether they could induce tubular morphogenesis by human microvascular endothelial cells in the co-culture model *in vitro*, and two cell lines, IN301 and IN157, were found to induce formation of tube-like structures in the collagen-containing gels. The roles of VEGF, bFGF and IL-8 in the glioma cell-induced angiogenesis are discussed with a view to the establishment of a human tumor angiogenesis model using human glioma cells.

MATERIALS AND METHODS

Materials The following materials were obtained from the indicated sources. TGFα was from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD). VEGF was donated by R & D Systems (Minneapolis, MN) and human recombinant bFGF by Oncogene Science Inc. (Uniondale, NY). Human VEGF cDNA was a kind gift from H. A. Weich (University of Freiberg, Germany), human bFGF cDNA was from D. B. Rifkin (New York University Medical Center, NY), human TGFα and TGFβ cDNAs were from R. Derynck (Genentech, Inc., South San Francisco, CA). IL-8 cDNA was prepared as described previously.[36] Anti-human VEGF neutralizing antibody, anti-bFGF antibody, and non-immune IgG were from R and D Systems, and anti-TGFα antibody was from Oncogene Science Inc. Anti-IL-8 antibody was prepared as described previously,[36] and the IgG fraction of one monoclonal antibody (WS-4) was used in this study.[37] These anti-bFGF and anti-TGFα antibodies at a concentration of 10 μg/ml almost completely antagonized tubulogenesis by vascular endothelial cells in the presence of 10 ng/ml bFGF and TGFα.[30, 32, 33] We obtained [α-^{32}P]dCTP (3000 Ci/mmol) from Amersham International plc (Buckinghamshire, UK).

Human microvascular endothelial cells Human microvascular endothelial cells were isolated from surgically removed omental adipose tissue as described.[31–35]

Cell lines Two human glioma cell lines, IN157 and IN301, derived from patients with glioma/astrocytoma were used in this study.[30, 38] These cell lines were obtained from M. Noble (University of London, UK). The glioma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 60 μg/ml kanamycin.[30, 38]

Northern blotting Northern blotting was performed as described previously.[30, 31, 39] Autoradiography was performed using Kodak XAR film.

Tube formation by vascular endothelial cells in type I collagen gels and quantitative analysis Human microvascular endothelial cells were plated onto the surface of type I collagen gel in M-199 containing 10% FBS. When the human microvascular endothelial cells reached confluence, the medium was replaced with M-199 containing 1% FBS and various growth factors, and the cells were incubated for 3 days. The medium was changed on the second day. On the third day, phase contrast microscopic

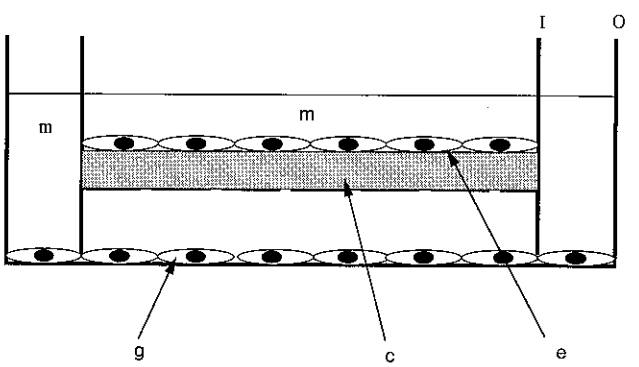


Fig. 1. Assay system for tube formation under co-culture. Each well consists of two chambers, an outer chamber (O; 38 ×7 mm) and an inner chamber (I; 30×7 mm). Glioma cells (g) were cultured in medium containing 10% FBS in the outer chamber, and at confluence, the medium was replaced with fresh medium. Separately, human microvascular endothelial cells (e) were seeded on type I collagen gel (c) in 2 ml of medium containing 10% FBS 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% in the medium (m) in both chambers, and the inner chamber was transferred into the outer chamber. After a 3-day incubation in the co-culture system, tube formation of human microvascular endothelial cells was determined.

pictures of each dish were recorded by a still video camera recorder (R5000H; Fuji, Tokyo), and the total length of tube-like structures per field was measured, using a Cosmozone IS image analyzer (Nikon, Tokyo), as described.[30, 32–35] Eight random fields per dish were measured, and the total length per field was calculated.

Assay of tube formation in the co-culture system We previously established an assay system to determine tube formation in vascular endothelial cells cultured with tumor cells in type I collagen gels.[30, 32–34] Here we assayed tube formation by human microvascular endothelial cells using this system (Fig. 1). Tumor cells were cultured in the outer chamber of six-well plates (each well, 38×7 mm; Corning Glass Works, Corning, NY) in 2 ml of DMEM containing 10% FBS. At confluence, the medium was exchanged for 2 ml of DMEM containing 1% FBS. Human microvascular endothelial cells were seeded separately in 2 ml of M-199 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 the inner chamber (30×7 mm). When the endothelial cells reached confluence, the FBS content of the medium was reduced from 10 to 1% and the inner chamber was transferred into the outer chamber. In this system, tubulogenesis should occur in endothelial cells in the collagen gel in the inner chamber, when tumor cells co-cultured in the outer chamber secrete angiogenic factors that pass through the filter of the

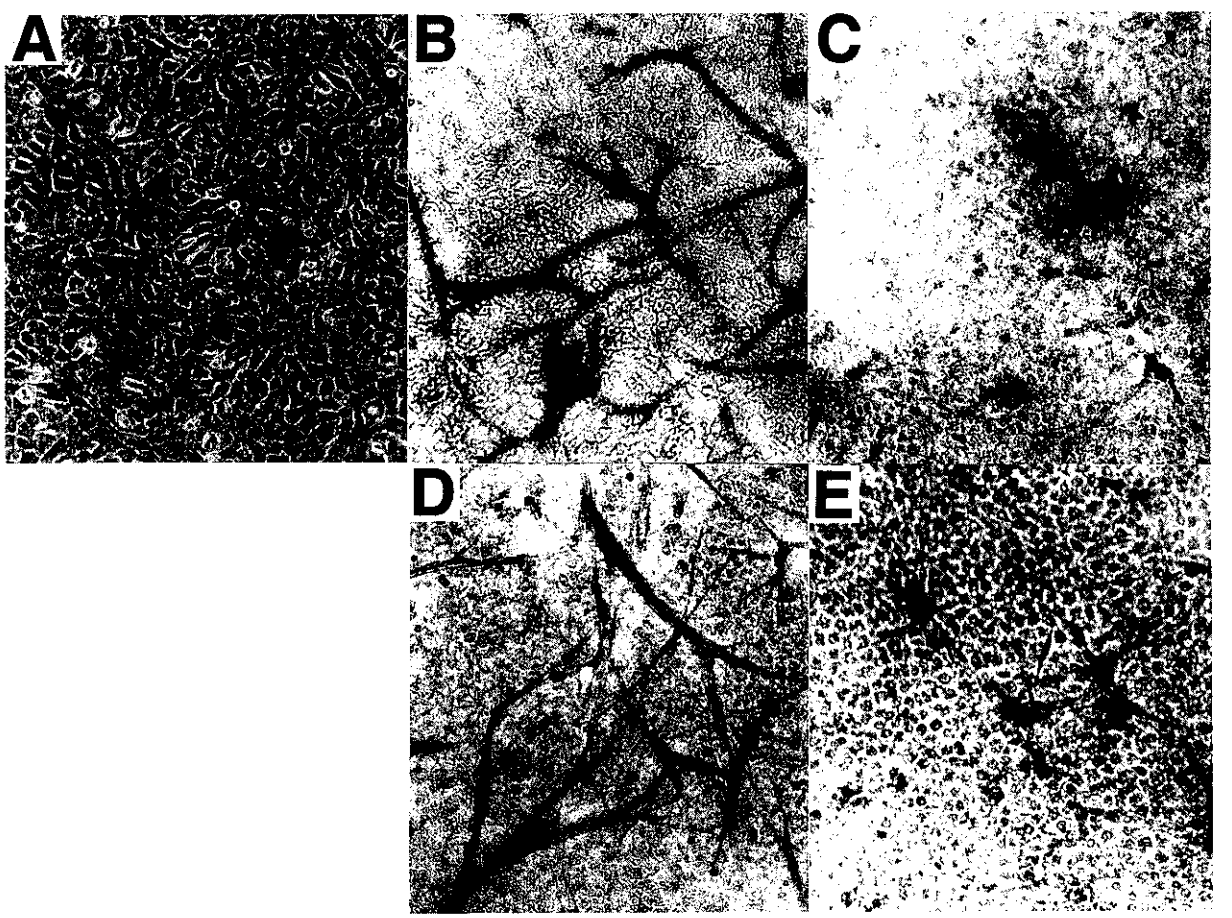
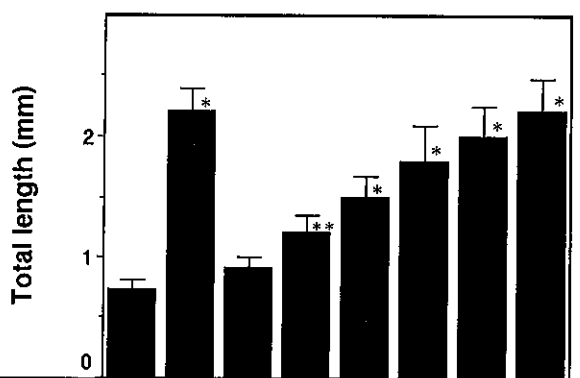


Fig. 2. Development of tube-like structures by human microvascular endothelial cells when co-cultured with glioma cells, and effect of antibodies against bFGF, VEGF and IL-8. Human microvascular endothelial cells were plated onto collagen gel when nearly confluent; at this point, the cells adopted a cobblestone-like appearance (A). Vessel-like structures appeared after a 3-day incubation in medium containing 1% serum, when co-cultured with IN301 cells in the absence (B) or presence of both anti-bFGF (10 μg/ml) and anti-VEGF (10 μg/ml) antibodies (C). Vessel-like structures also appeared on co-culture for 3 days with IN157 cells in the absence (D) or presence of 10 μg/ml anti-IL-8 antibody (E). Before photography, cells in (B)–(E) were fixed and stained briefly in modified May-Gruenwald's solution to demonstrate tube-like structures formed by microvascular endothelial cells. Magnification ×400.

inner chamber. After a 3-day incubation, tube formation in the endothelial cells was quantitated by recording on a floppy video disk. The total length of tube formation was analyzed using the Cosmozone program (NEC PC-9801; NEC, Tokyo).

RESULTS

Human microvascular endothelial cells extracted from omentum adipose tissues not only develop tube-like structures in type I collagen gel in the presence of EGF/TGFα or HB-EGF,[32-35] but also capillary networks on Matrigel.[40] Microvascular endothelial cells assumed a typical cobblestone-like appearance on type I collagen gel in the absence of growth factor (Fig. 2A). Endothelial cells cultured for 3 days with both 10 ng/ml bFGF and 100 ng/ml VEGF and with 10 ng/ml IL-8 produced branching vessel-like structures inside the gel matrix (data not shown). To measure the effect of the growth factors on tube formation in type I collagen gel, we determined the total length of the tube-like structures by image analysis. In the absence of growth factors, tube-like structures in the collagen gel did not develop. However, after a 3-day incubation with 10 ng/ml TGFα, marked development of tube-like structures (* $P<0.01$) was seen (Fig. 3). The total tube length was about 0.72±0.09 mm and 2.2±0.2 mm in the absence and presence, respectively, of TGFα at 10 ng/ml. The addition of 10 ng/ml bFGF, which is a potent tubulogenic factor in bovine endothelial cells, induced a slight formation of tube-like structures. The presence of bFGF at 100 ng/ml did not further increase the tubulogenesis activity (data not shown). By contrast, exogenous VEGF stimulated the development of tube-like structures. The total tube length was about 1.2±0.15 mm and 1.5±0.2 mm in the presence of 10 ng/ml and 100 ng/ml VEGF alone, 1.8±0.4 mm in 10 ng/ml of both VEGF and bFGF, and 2.0±0.3 mm in 10 ng/ml bFGF and 100 ng/ml VEGF (Fig. 3). The total length of tube-like structures was found to be 2.2±0.27 mm in the presence of IL-8 at 10 ng/ml (Fig. 3). In comparison with the outcome in the absence of any factor, microvascular endothelial cells significantly developed tube-like structures when incubated with VEGF alone or with both VEGF and bFGF. Although VEGF or a combination of VEGF and bFGF exerted weaker tubulogenic activity than TGFα, human microvascular endothelial cells also appeared to be susceptible to VEGF in the absence or presence of bFGF.



TGFα (ng/ml)	0	10	0	0	0	0	0	0
bFGF (ng/ml)	0	0	10	0	0	10	10	0
VEGF (ng/ml)	0	0	0	10	100	10	100	0
IL-8 (ng/ml)	0	0	0	0	0	0	0	10

Fig. 3. Development of tube-like structures by vascular endothelial cells in the presence of TGFα, bFGF, VEGF and IL-8. Endothelial cells were plated onto collagen gel and incubated in medium containing 10% FBS. On the following day, the medium was replaced with that containing 1% FBS together with the indicated doses of TGFα, bFGF, VEGF and IL-8. After 3 days, 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 were measured and the total length per field was calculated. Columns are means±SD (bars) of triplicate experiments. Statistically significant differences (* $P<0.01$ and ** $P<0.05$) in comparison with the absence of growth factors are indicated.

Glioma cell lines express various levels of mRNAs for growth factors, proteinases and proteinase inhibitors.[38] We first determined the cellular mRNA levels of VEGF, bFGF, TGFα, TGFβ and IL-8 in IN157 and IN301. We found that those of VEGF, bFGF and TGFβ were 3- to 5-fold higher in IN301 than in IN157, whereas the level of IL-8 was much higher in IN157 than in IN301 (Fig. 4). The level of TGFα was similar between IN157 and IN301 (Fig. 4). Determination of IL-8 by enzyme-linked immunosorbent assay using a monoclonal antibody against IL-8 demonstrated that IN157 and IN301 cells secreted 2920 pg/ml and 31 pg/ml IL-8, respectively, into the medium (M. Ryuto and M. Kuwano, unpublished data). However, we did not examine the levels of other factors, VEGF, TGFα and bFGF.

Using the co-culture model of glioma cell-dependent angiogenesis, we examined whether co-cultured glioma cells produced TGFα, bFGF and VEGF, resulting in the further development of tube-like structures by human microvascular endothelial cells. Endothelial cells produced branching tube-like structures inside the gel matrix when co-cultured with IN301 (Fig. 2B), and co-administration of both anti-bFGF and anti-VEGF antibodies inhibited the development of the tube-like structures (Fig. 2C). Determination of the total length of tube-like structures indicated that co-cultured IN301 apparently enhanced the formation of these structures (Fig. 5A).

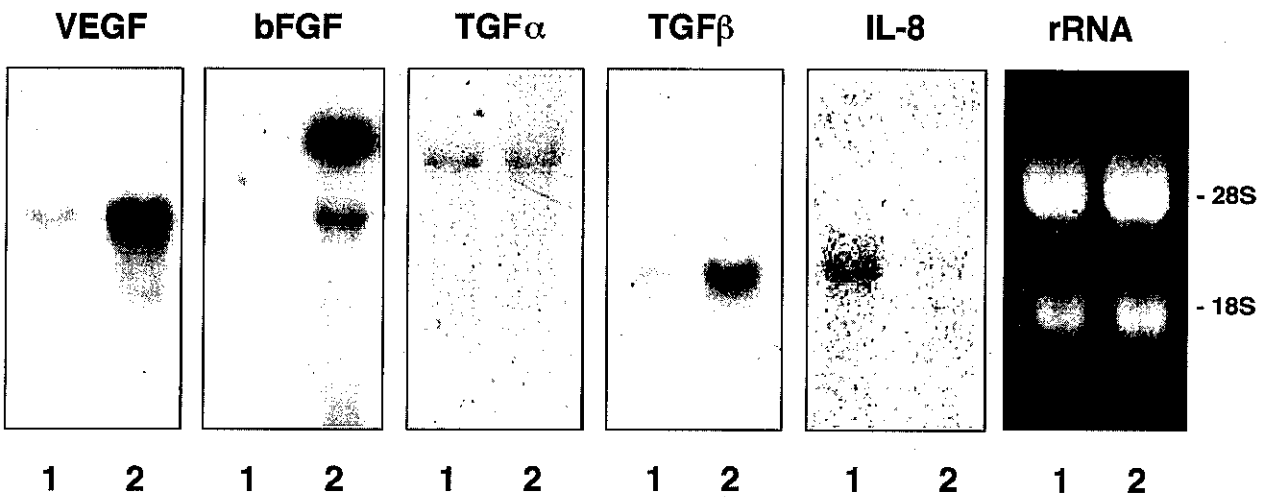


Fig. 4. Northern blots of VEGF, bFGF, TGFα, TGFβ and IL-8 mRNA in the glioma cell lines IN157 (lane 1) and IN301 (lane 2). RNA extracted from the glioma cells was hybridized to ^{32}P-labeled cDNA probes for the 5 growth factors in Hybrisol. Autoradiography proceeded using Kodak XAR film exposed for 3 days. Control RNA samples are also shown.

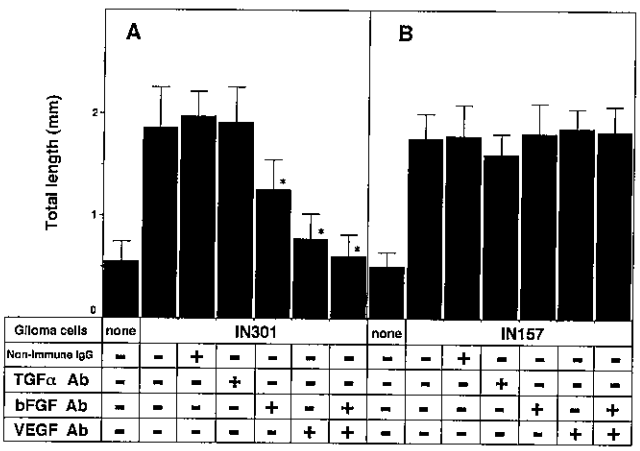


Fig. 5. Tube formation by endothelial cells co-cultured with IN301 or IN157 cells and the effect of antibodies against TGFα, bFGF and VEGF. Vascular endothelial cell tube formation was examined in the presence of 1% serum with or without co-culture for 3 days. During this assay, antibody against TGFα, VEGF or bFGF at 10 μg/ml was incubated with co-cultured glioma cells for 3 days. Non-immune serum was used as the control. On the 3rd day of co-culture, tube formation was quantified. Statistically significant differences ($*$ $P<0.01$) from the control with co-cultured cells in the absence of antibody are indicated.

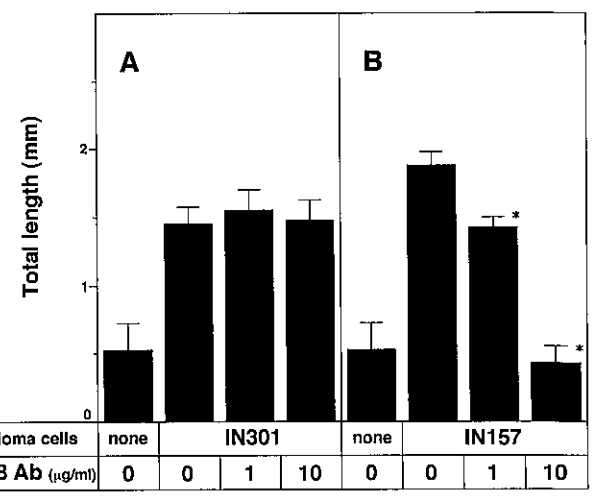


Fig. 6. Tube formation by endothelial cells co-cultured with IN301 or IN157 cells, and the effect of antibody against IL-8. Vascular endothelial cell tube formation was examined in the presence of 1% serum with co-culture for 3 days in the presence of antibody against IL-8 at 1 or 10 μg/ml. On the 3rd day of co-culture, tube formation was quantified. Statistically significant differences ($*$ $P<0.01$) from the control with co-cultured cells in the absence of antibody are indicated.

This tubulogenesis activity of IN301 was partly blocked by an antibody against bFGF and almost completely blocked by anti-VEGF antibody. The inhibition of tubulogenesis was even more pronounced when both anti-bFGF and anti-VEGF antibodies were present (Fig. 5 A). By contrast, there was no inhibition in the presence of anti-TGFα antibody. Although IN157 cells also promoted development of tube-like structures by endothelial cells at similar levels to those of IN301 cells, tubulogenesis was not inhibited by anti-TGFα, anti-bFGF, or anti-VEGF antibodies (Fig. 5B).

Co-cultured IN157 apparently promoted formation of tube-like structures inside the gel matrix (Fig. 2D), and co-administration of anti-IL-8 antibody was found to inhibit the IN157-induced tubular morphogenesis by microvascular endothelial cells (Fig. 2E). As seen in Fig. 6, exogenous addition of anti-IL-8 antibody at 1 and 10 μg/ml did not inhibit the IN301-induced tubular morphogenesis, whereas co-administration of anti-IL-8 antibody at 10 μg/ml almost completely inhibited the IN157-induced tubular morphogenesis. Anti-IL-8 antibody at 1 μg/ml partially inhibited the IN157-induced tubular morphogenesis. Like the other antibodies used here (see "Materials and Methods"), anti-VEGF antibody and anti-IL-8 antibody at 10 μg/ml also almost completely antagonized tubulogenesis by vascular endothelial cells in the presence of 10–100 ng/ml VEGF, and also 1–30 ng/ml IL-8 (data not shown). These findings suggested that IN301-dependent tubulogenesis was mediated through VEGF and bFGF, and that IN157-induced tubulogenesis proceeded mainly through IL-8.

DISCUSSION

We have established an *in vitro* model for tumor angiogenesis by human glioma cells, which produce high levels of bFGF and induce tubular morphogenesis in bovine aortic endothelial cells.[30] In that assay, however, we used bovine aortic endothelial cells, which are highly susceptible to bFGF. In this study, we used human microvascular endothelial cells in the tumor angiogenesis model of human glioma cells. In the *in vitro* angiogenesis model with human microvascular endothelial cells, EGF/TGFα or HB-EGF enhances cell migration, chemotaxis, plasminogen activator production and tube formation in collagen gel.[32–35] The differential sensitivities of bovine aortic endothelial cells and human microvascular endothelial cells to bFGF and EGF or its family members might be due to a difference in cellular levels of their receptors, since cellular expression of bFGF receptors was found to differ between bovine and human endothelial cells (unpublished data). This study also showed that not only TGFα, but also VEGF or both VEGF and bFGF, promoted tubular morphogenesis by human microvascular endothelial cells. Angiogenesis by human microvascular endothelial cells thus appeared to be susceptible to VEGF alone or in combination with bFGF. Although bFGF alone only weakly induced tubular morphogenesis by human microvascular endothelial cells in this study, we confirmed the synergistic effect of VEGF and bFGF upon angiogenesis *in vitro*. TGFα appeared to be a more potent inducer of tube formation by human microvascular endothelial cells than VEGF or bFGF, while the IL-8-induced tubular morphogenesis in the *in vitro* model was similar to that of TGFα (Fig. 3).

In the angiogenesis model *in vitro*, human microvascular endothelial cells on type I collagen gel in the inner chamber are co-cultured with human glioma cells in the outer chamber. One glioma cell line, IN301, had much higher mRNA levels of VEGF, bFGF and TGFβ than those of the other glioma cell line, IN157, whereas the two had similar TGFα mRNA levels. Numerous vessel-like structures developed during co-culture with IN301 or IN157 cells, and the total length of the tube-like structures increased about 3-fold in both cell lines. Human microvascular endothelial cells express VEGF (Flt-1) and bFGF receptors (bek, flg) (unpublished data), as well as EGF receptors.[33, 35] Furthermore, the IN301-induced tube formation in microvascular endothelial cells was inhibited markedly by anti-VEGF antibodies, partly by anti-bFGF antibody, and almost completely by antibodies against both VEGF and bFGF. Anti-TGFα antibody can completely block the tubulogenesis induced by TGFα-expressing human esophageal cancer cells or keratinocytes,[32, 33] but it cannot inhibit both IN301- and IN157-induced tubulogenesis, suggesting that the involvement of TGFα in IN301- or IN157-dependent tubulogenesis is less likely. As an underlying mechanism, IN301 cells secrete VEGF and also bFGF, which may induce paracrine control of tubulogenesis in vascular endothelial cells. The paracrine control of angiogenesis by tumor cells may be mediated through VEGF and/or bFGF. By contrast, the TGFβ mRNA level was much higher in IN301 than in IN157 (Fig. 4). TGFβ shows angiogenic or anti-angiogenic activities in various angiogenesis systems.[3, 5, 6] In the microvascular endothelial cell system, TGFβ shows inhibition of tubular morphogenesis and plasminogen activator synthesis,[41] suggesting that an involvement of TGFβ in IN301-dependent tubular morphogenesis by microvascular endothelial cells is less likely.

In this model, one would also expect tubular morphogenesis by microvascular endothelial cells when cultured with a human glioma cell line producing high levels of TGFα. Most human glioma cell lines appear not to produce large amounts of TGFα, but one cell line (U251) among the 7 does do so.[30] U251 cells could induce formation of tube-like structures by human microvascular endothelial cells, but U251-dependent tubular morphogenesis appeared to be less than that with IN157 or IN301 (unpublished data).

IN157-induced tubulogenesis was not inhibited in the presence of antibodies developed against VEGF, bFGF, and TGFα, but antibody developed against IL-8 inhibited the IN157-induced tubular morphogenesis. IN157 cells expressed much higher IL-8 mRNA levels than IN301 cells. A paracrine control through secretion of IL-8 from IN157 cells appears to be partly involved in tubular morphogenesis by human microvascular endo-

thelial cells. IL-8, which is often abunduntly produced in brain tumor tissues and glioma cells,[14–16] induces chemotaxis of human umbilical cells and angiogenesis in rat cornea.[17] Further study is required to determine whether IL-8 plays a major role in human glioma-induced angiogenesis in the brain.

In accordance with the animal angiogenesis model of gliomas,[21, 28] our present study indicates that glioma cells producing high levels of VEGF induce tube formation *in vitro*. We also reported recently that vasculature in human clinical glioma samples is more closely correlated with expression of VEGF, than with that of bFGF, TGFα or TGFβ.[24] Expression of VEGF is thus expected to induce tube formation in various angiogenesis models as well as in clinical brain samples. Both the *in vitro* angiogenesis model with bovine aortic endothelial cells and the *in vivo* model in mice have demonstrated that bFGF is mainly involved in human glioma cell-induced angiogenesis.[30] In contrast, an *in vitro* model with human microvascular endothelial cells revealed that EGF/TGFα or HE-EGF is a potent tubulogenic factor.[32–35] However, human glioma cells producing high levels of TGFα fail to induce angiogenesis in mice.[30] The protein SPARK induces tube formation *in vitro*, but not *in vivo* unless specific internal peptides are released from it by proteolysis.[42] These findings thus suggest that not all results obtained by induction of tube formation *in vitro* will apply to induction of angiogenesis *in vivo*.

In conclusion, our tumor angiogenesis model for gliomas suggests that there are at least two regulatory pathways by means of which various angiogenic factors induce their signals. One is mediated through paracrine control by VEGF and/or bFGF, whereas the other is mediated through IL-8. Our tumor angiogenesis model may be useful for understanding the underlying mechanism and factors involved in the angiogenesis of human vascular endothelial cells caused by human glioma cells.

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