Acta Neuropathol (1998) 96 : 322–328

REGULAR PAPER

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Heparin-binding epidermal growth factor-like growth factor stimulates mitogenic signaling and is highly expressed in human malignant gliomas

Received: 18 November 1997 / Revised, accepted: 10 March 1998

Abstract We previously reported that schwannoma-derived growth factor (SDGF), a member of heparin-binding epidermal growth factor (EGF) family, participates in autocrine pathways and promotes rat glioma cell growth. To investigate the potential role of similar molecules in human gliomas, we examined 7 human glioma cell lines and 11 glioblastoma specimens for expression of the human homologue of SDGF, amphiregulin (AR), as well as heparin-binding EGF-like growth factor (HB-EGF). Northern blot analysis revealed that only one cell line and no tumor specimens expressed AR mRNA. In contrast, HB-EGF mRNA was expressed in all human glioma cell lines and its level of expression was two- to five-fold higher than that of control brain tissues in 8 of 11 glioblastoma cases. Immunohistochemistry demonstrated that membrane-anchored HB-EGF (proHB-EGF) and EGFR were co-expressed in 44% of 34 human malignant gliomas. Introduction of exogenous HB-EGF (10 ng/ml) increased human glioma cell proliferation, and anti-HB-EGF blocking antibodies reduced the growth of glioma cells by 30–40%, confirming the presence of an autocrine loop. When added to the medium, transforming growth factor-α, basic fibroblast growth factor, or HB-EGF rapidly induced HB-EGF mRNA expression. These results indicate that HB-EGF and proHB-EGF contribute to the growth of human malignant glioma cells, most likely through autocrine and juxtacrine mechanisms.

Key words Heparin-binding epidermal growth factor-like growth factor · Autocrine · Juxtacrine · Glioma

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Introduction

Growth factors and their cognate receptors have been implicated in the transformation or proliferation of a variety of neoplasms, including gliomas [40]. Glioblastomas frequently amplify and overexpress the gene encoding epidermal growth factor receptor (EGFR) [17, 21, 36, 40, 41], suggesting that EGFR confers a growth advantage to glioma cells. Several EGF-related growth factors have also been identified and the potential roles of these proteins in tumorigenesis are currently under investigation [2, 11, 18, 32, 37].

We previously demonstrated that schwannoma-derived growth factor (SDGF), a member of the heparin-binding EGF family [18], is frequently overexpressed in rat glioma cells and may form an autocrine loop to stimulate proliferation [24]. In human gliomas, however, the role of heparin-binding EGF family members is not fully understood. These observations prompted us to investigate the potential roles of two human proteins which belong to this relatively new growth factor family, amphiregulin (AR) [32] and heparin-binding EGF-like growth factor (HB-EGF) [11]. AR is the human homologue of rat SDGF [32], and HB-EGF is another member of heparin-binding growth factors implicated in tumorigenesis in hepatomas [15], gastric carcinomas [26], and pancreatic cancers [20]. We speculated these proteins may also be involved in glioma formation, in a manner analogous to that observed in rat gliomas.

Materials and methods

Cell lines and primary tumors

Human glioma cell lines SF126, SF188, SF763, U87MG, U251MG, and U343MG were kind gifts from Dr. Mark Rosenblum (Henry Ford Hospital, Detroit, Mich.). Glioma cell line T98G was obtained from the American Type Culture Collection (Rockville, Md.). All cell lines were cultured in standard medium consisting of Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 300 μg/ml glutamine, 0.14% sodium hydrogen carbonate, 100 μg/ml streptomycin and 100 units/ml penicillin G (Life Technologies, Grand Island, N.Y.).

Tissue specimens of glioblastomas were snap frozen in liquid nitrogen immediately after resection and stored at –80°C until use. In two cases, after informed consent was obtained, RNA samples were extracted from areas without any significant degree of tumor infiltration, and were used as normal control.

Recombinant human HB-EGF (rHB-EGF) was provided by Dr. Judith A. Abraham (Scios Nova, Mountain View, Calif.). Recombinant human EGF was purchased from Gibco BRL (Tokyo, Japan), basic fibroblast growth factor (bFGF) and transforming growth factor-α (TGF-α) were purchased from Oncogene Science (Cambridge, Mass.), and insulin-like growth factor (IGF-I) was from Austral Biologicals (San Ramon, Calif.).

Cell growth assay

Glioma cells were seeded in six-well plates at a density of 4×10^4 cells/well and were cultured for 8 h in standard medium. The cells were then incubated in serum-free standard medium containing various amounts of human rHB-EGF, goat anti-HB-EGF specific polyclonal antibody 197 (45 μg/ml) [10] or normal goat IgG (45 μg/ml) for 24 h. The culture medium was replaced every 24 h with fresh serum-free standard medium containing rHB-EGF or anti-HB-EGF antibody. After 72 h, the cells were trypsinized and living cells were counted by the trypan blue dye exclusion method. The growth of each glioma cell line was assayed in triplicate.

Northern blot analysis

RNA extraction and Northern blot analysis were performed as previously described [24]. HB-EGF cDNA and AR cDNA were labeled with [^{32}P]dCTP by random-primed labeling, and used as probes. Loading of RNA was checked by reprobing the blot with a cDNA probe to GAPDH.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues from 34 malignant gliomas (9 anaplastic astrocytomas and 25 glioblastomas) were immunostained for HB-EGF and EGFR. Primary antibodies used in this study were polyclonal rabbit anti-human HB-EGF antibody (H1, 1:200), which specifically detects membrane-anchored proHB-EGF [27], and mouse monoclonal anti-EGFR antibody (1:20, NCL-EGF, Novocastra, Claremont Place, UK), which detects the extracellular domain of EGFR protein. Antibody for proHB-EGF has been proven to be specific when used for immunohistochemistry [15]. Following deparaffinization and rehydration, the tissue sections (5 μm) were transferred into a pressure cooker containing 0.01 M sodium citrate solution (pH 6.0), and were treated for 5 min at maximum pressure [29]. After rinsing with PBS, the tissues were incubated with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase. The sections were blocked for 30 min with 10% goat serum (for HB-EGF) or 10% horse serum (for EGFR), rinsed in PBS, and incubated with the primary antibody at room temperature for 2 h. The sections were then washed with PBS and incubated with an appropriate secondary biotinylated antibody (1:1000, Vector Lab., Burlingame, Calif.) for 30 min. After several washes with PBS, products were visualized using the ABC kit (Vector Lab.) and diaminobenzidine as chromogen. Hematoxylin was used as counterstain. The primary antibodies were replaced with corresponding normal serum for negative controls.

Results

Expression of HB-EGF and AR by glioma cells

Of the 7 glioma cell lines and 11 glioblastomas examined, only 1 cell line, SF763, expressed a single 4.1-kb AR transcript. No other cell lines or glioblastomas expressed AR at a detectable level. In contrast, all 7 glioma cell lines expressed three distinct HB-EGF transcripts (2.5, 1.5, and 1 kb) (Fig. 1A), as previously described in human macrophages [11]. The 2.5-kb HB-EGF mRNA was a major transcript in U87MG, SF763, and T98G cells and was expressed

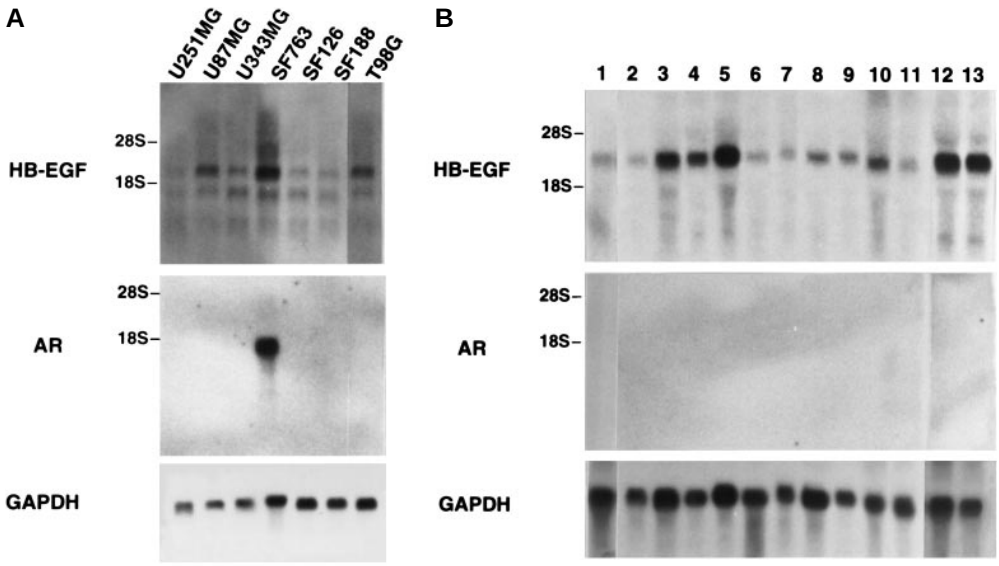

Fig. 1 A, B Northern blot analysis of human malignant gliomas. **A** HB-EGF and AR mRNA expression in human malignant glioma cell lines. Total RNA was electrophoresed and transferred to nylon filters. The membranes were then hybridized with ^{32}P-labeled HB-EGF and AR cDNA probes. To confirm equivalent loading of the lanes, the membranes were rehybridized with a GAPDH cDNA probe. The positions of the 18 S and 28 S ribosomal RNA bands are shown as size markers. **B** HB-EGF and AR mRNA expression in glioblastoma tissues. *Lanes 1* and *2* Control nonmalignant brain tissues, *lanes 3–13* glioblastomas (*EGF* epidermal growth factor, *HB-EGF* heparin-binding EGF-like growth factor, *AR* amphiregulin)

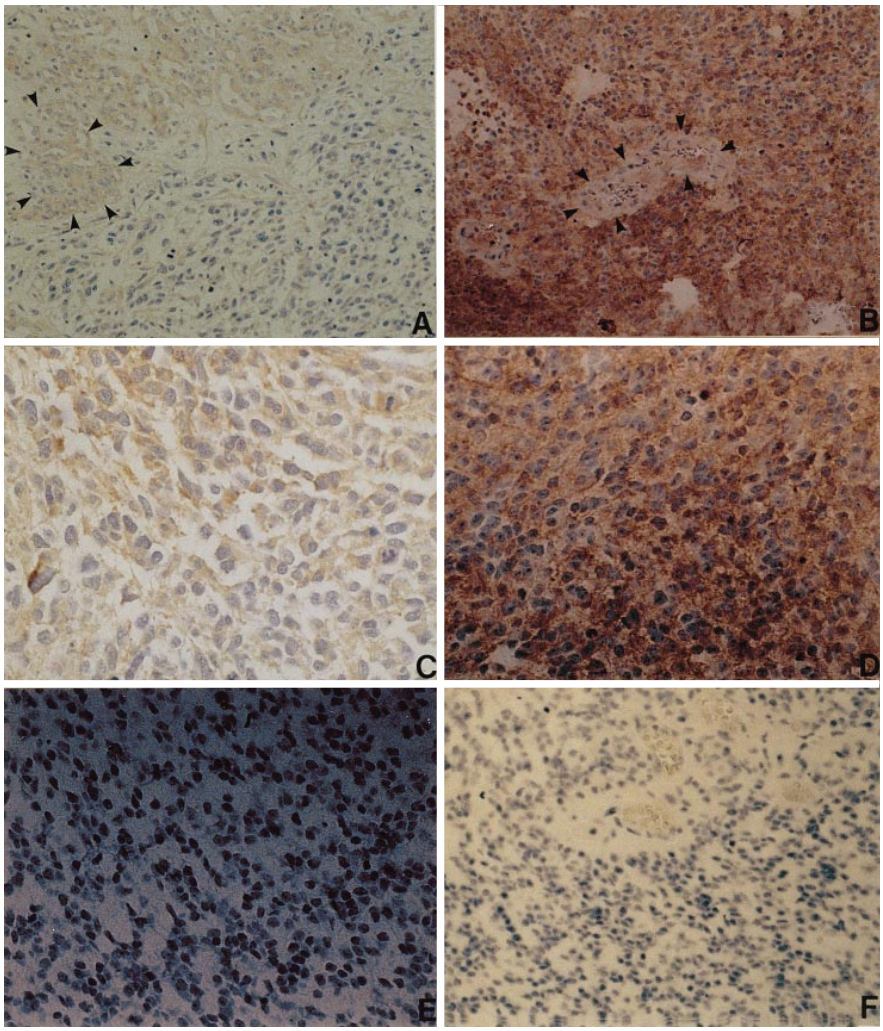

Fig. 2 A–F Immunohistochemical staining of proHB-EGF and EGFR in tissue sections from glioblastomas. **A**, **C** ProHB-EGF immunoreactivity was evident in the cytoplasm of neoplastic cells and proliferating vascular endothelial cells (*arrowheads*). **B**, **D** Intense staining of EGFR was evident in the cytoplasm and plasma membranes of neoplastic cells, but no expression was evident in the area of endothelial proliferation (*arrowheads*). **E**, **F** As negative controls, normal rabbit serum (**E**) or normal mouse IgG (**F**) was used in place of the primary antibody. (*EGFR* EGF receptor). **A**, **B**, **E**, **F** × 200; **C**, **D** × 400

Table 1 Expression of proHB-EGF and EGFR in 34 malignant gliomas (*HB-EGF* heparin-binding epithelial growth factor-like growth factor, *EGFR* EGF receptor)

Type of tumor	Both proHB-EGF and EGFR	ProHB-EGF only	EGFR only	Neither
Anaplastic astrocytoma (n = 9)	2	2	1	4
Glioblastoma (n = 25)	13	4	0	8
Total	15	6	1	12

at levels three to five times higher than in U251MG, U343MG, SF126, and SF188 cells. We confirmed the expression of proHB-EGF protein in glioma cell lines by cell surface biotinylation and immunoprecipitation with anti-human HB-EGF antibody (H1) (data not shown). The 2.5-kb HB-EGF transcript was increased two- to fivefold in 8 of 11 glioblastoma cases by comparison with the control human brain tissues (Fig. 1B).

Expression of HB-EGF and EGFR in human glioma tissues

We next determined whether human malignant glioma cells exhibit HB-EGF immunoreactivity and whether malignant gliomas co-express HB-EGF and EGFR *in vivo*. A representative case with positive immunohistochemical staining for both EGFR and HB-EGF is shown in Fig. 2. Immunostaining with anti-EGFR antibody demonstrated intense immunoreactivity localized to the cell membrane and cytoplasm (Fig. 2D) in 3 of 9 (33%) anaplastic astrocytomas and 13 of 25 (52%) glioblastomas. No reactivity was observed in vascular endothelial cells (Fig. 2B), or in adjacent normal brain. Immunostaining with the anti-proHB-EGF-specific antibody (H1) demonstrated intense immunoreactivity in the cytoplasm of tumor cells, as well as in the endothelial cells of proliferating capillaries in gliomas (Fig. 2A, C). Two of 9 (22%) anaplastic astrocytomas and 13 of 25 (52%) glioblastomas stained positive for both proHB-EGF and EGFR proteins (Table 1). ProHB-EGF was expressed in 2 of 3 EGFR-positive anaplastic astrocytomas and 13 of 13 EGFR-positive glioblastomas

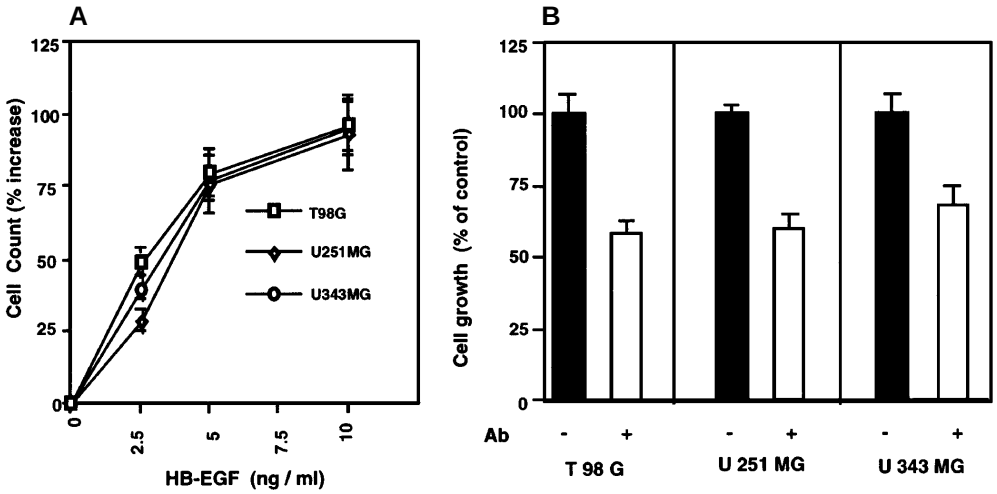

Fig. 3 A, B Effect of HB-EGF on the proliferation of human glioma cells. **A** Effect of human recombinant (r) HB-EGF on glioma cell growth. The results are expressed as the percentage of treated cells relative to control cells, calculated as $[(a–b)/b] \times 100$, where a and b are the numbers of treated and control cells, respectively. The cell numbers for control cultures were 14.4×10^4 for T98G, 12.6×10^4 for U251MG, and 10.4×10^4 for U343MG. Each value represents the mean ± SD of triplicate determinations. **B** Growth inhibition of glioma cells by anti-HB-EGF blocking antibody. The results are expressed as a percentage of the number of treated cells relative to the number of cells in control cultures without serum (100%). The cell numbers for control cultures were 14.0×10^4 for T98G, 13.3×10^4 for U251MG, and 11.2×10^4 for U343MG. Each value represents the mean ± SD of triplicate determinations

(Table 1). ProHB-EGF and EGFR were co-expressed in 15 of 34 (44%) malignant human gliomas, and neither protein was expressed in 12 (35%). This association was statistically significant ($P = 0.004$, Fisher's exact test). Of the 18 EGFR-negative tumors, 2 anaplastic astrocytomas and 4 glioblastomas were positive for proHB-EGF. Only 1 of 16 (6%) EGFR-positive tumors was negative for proHB-EGF.

Effect of rHB-EGF on human malignant glioma cell lines

Next, we examined the effect of HB-EGF on glioma cell proliferation. Three glioma cell lines (T98G, U251MG, and U343MG) were cultured independently for 72 h with rHB-EGF at various concentrations ranging from 2.5 to 10 ng/ml. All three cell lines increased in number in a dose-dependent manner (Fig. 3A).

To determine if HB-EGF stimulates growth in an autocrine manner, we examined the effect of anti-HB-EGF blocking antibody [12]. When added to the medium, anti-HB-EGF blocking antibody (45 μg/ml) reduced proliferation by 30–40% as compared to normal goat IgG at the same concentration after a 3-day incubation (Fig. 3B). These findings indicated that HB-EGF acts as an autocrine growth factor for human glioma cells.

Induction of HB-EGF gene expression by glioma mitogens

Some growth factors are known to induce expression of their own (autoinduction) as well as related growth factors (cross-induction) [1, 5]. Therefore, we examined the effect of rHB-EGF on HB-EGF mRNA expression in human glioma cells. Addition of rHB-EGF (10 ng/ml) increased HB-EGF mRNA expression in T98G glioma cells 3-fold after 1 h of incubation, and 10-fold after 3 h (Fig. 4). Since TGF-α and EGF are members of the EGF family and share the same receptor with HB-EGF, we further examined their effects on HB-EGF mRNA expression. In T98G cells, EGF (10 ng/ml) increased HB-EGF mRNA expression 15-fold at 3 h and 5-fold at 6 h, while TGF-α (10 ng/ml) increased 5-fold at 3 h and 3-fold at 6 h. We also tested TGF-β, bFGF, and IGF-I, which are not members of the EGF family, for their ability to stimulate HB-EGF mRNA expression in human glioma cells. Northern blotting demonstrated that bFGF increased HB-EGF mRNA expression 7-fold at 3 h and 3-fold at 6 h in T98G cells. However, IGF-I and TGF-β at the same concentration had little stimulative effect on HB-EGF expression in the tumor cells. Similar results were obtained with U251MG and U343MG cells (data not shown).

Discussion

Our data presented here strongly indicate that HB-EGF contributes, at least in part, to the growth of malignant gliomas. HB-EGF was expressed in all the glioma cell lines examined and was also highly expressed in a significant number of human glioblastoma tissues at the RNA level. Exogenous HB-EGF stimulated the growth of human glioma cells *in vitro*, and neutralizing anti-HB-EGF antibody suppressed the proliferation of glioma cells. These findings indicate that HB-EGF may participate in the autocrine growth of human malignant glioma cells. HB-EGF is a 20–22-kDa glycoprotein that was originally cloned from macrophage-like U937 cells [11]. It is synthesized as a pro-form (proHB-EGF) that is integrated into

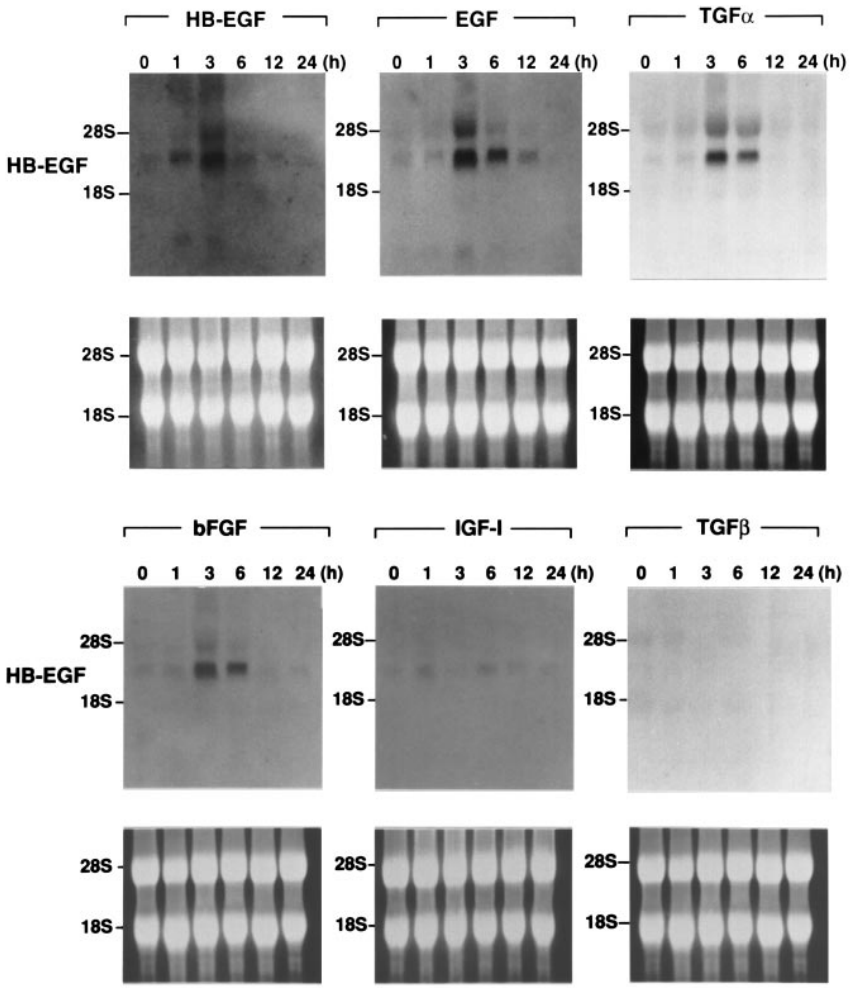

Fig. 4 Effect of HB-EGF and various growth factors on HB-EGF mRNA expression in T98G glioma cells. Cells were cultured for 48 h in serum-free medium and were then incubated with rHB-EGF, EGF, TGF-β, bFGF, IGF-I, or TGF-α (all 10 ng/ml) for the indicated periods. Total RNA (20 μg) from human glioma cells incubated with a growth factor was fractionated by electrophoresis in a 1% agarose/formaldehyde gel, transferred to a nylon membrane, and probed with ^{32}P-labeled HB-EGF cDNA. The quality and quantity of RNA applied on a gel were confirmed by staining with ethidium bromide. The positions of the 18 S and 28 S ribosomal RNA bands are shown as size markers. All experiments were repeated with a different set of RNA from independent experiments (*TGF* transforming growth factor, *bFGF* basic fibroblast growth factor, *IGF* insulin-like growth factor)

the plasma membrane and then processed by proteolytic cleavage to a soluble form of 76–87 amino acid residues [12]. Soluble HB-EGF activates the EGFR and stimulates growth in smooth muscle cells, keratinocytes, hepatocytes, and fibroblasts [10, 11, 13, 16], but similar effects on gliomas have not been described to date. In addition, our results show that HB-EGF expression is induced by HB-EGF, bFGF, EGF, or TGF-α in human glioma cells *in vitro*, as previously observed in human keratinocytes and vascular smooth muscle cells [7, 10]. Since human glioma cells produce TGF-α and bFGF [9, 28, 35], these observations raise the possibility that multiple tumor-derived growth factors may mutually regulate their signal transduction pathways and play an important role in stimulating the growth of glioma cells. It is also possible that cross-induction by these growth factors might lead to an overexpression of HB-EGF in glioblastomas.

HB-EGF expressed in primary gliomas is apparently the membrane-anchored form proHB-EGF, as indicated by immunohistochemistry. Membrane-anchored proHB-EGF has been shown to stimulate cell growth and survival of EGFR-positive cells by a direct cell-cell contact, probably through a juxtacrine mechanism [14, 25]. Interestingly, our data showed a strong association between proHB-EGF and EGFR expression in malignant gliomas, suggesting that a similar mechanism may exist in gliomas *in vivo*.

Malignant gliomas are among the most neovascularized neoplasms [4, 6, 19, 31] and a variety of growth factors are known to be produced by gliomas and stimulate angiogenesis including bFGF, PDGF, TGF-α and vascular endothelial growth factor (VEGF), among which VEGF is regarded as a major factor [3, 22, 23, 30, 31, 33, 34]. Although HB-EGF has no mitogenic activity on vascular endothelial cells [11], we found it was highly expressed in proliferating endothelial cells in glioma tissues. It has been shown that HB-EGF possesses tubulogenic activity on human microvascular endothelial cells in vitro [39], and our preliminary data indicate that HB-EGF, like EGF and bFGF [8, 38], stimulates production and secretion of VEGF by glioma cells in vitro (unpublished data). Therefore, it is possible that HB-EGF co-operates with those growth factors and contributes to angiogenesis in an indirect manner, although the extent of contribution is yet to be determined.

In conclusion, the present study demonstrates that HB-EGF is frequently expressed in human glioma cells and glioblastomas, and may play significant roles in glioma growth. It is possible, therefore, that modalities aimed at disrupting HB-EGF-dependent pathways may ultimately have a role in the treatment of malignant gliomas.

Acknowledgements We wish to thank Drs. K. Ueki (Department of Neurosurgery, Faculty of Medicine, University of Tokyo) and K. Knudsen (Ludwig Institute for Cancer Research, San Diego Branch) for helpful comments on the manuscript. This study was supported by a Grant-in-Aid from the Ministry of Health and Welfare of Japan for the 2nd-term Comprehensive 10-Year Strategy for Cancer Control and by grants from the Ministry of Health and Welfare of Japan, and from the Ministry of Education, Science and Culture of Japan for Cancer Research to Y.K.

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