Jpn. J. Cancer Res. 88, 564–577, June 1997

Modulation of Motility and Proliferation of Glioma Cells by Hepatocyte Growth Factor

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Invasive proliferation is a critical biological characteristic of gliomas. We evaluated the activities of hepatocyte growth factor (HGF) on proliferation and motility of glioma cells, comparing them with the effects of other growth factors (EGF, bFGF, PDGF-BB, TGF-β1). Seven primary culture lines all expressed c-*met* and HGF mRNA, and secreted HGF. HGF stimulated ^{3}H-thymidine uptake of every glioma cell line (30 to 70% upregulation). Boyden chamber assay and scattering assay revealed that HGF promoted cell motility with chemokinetic and strong chemotactic activities. Concentric circle assay showed that HGF promoted two-dimensional expansion (proliferation and motility) most strongly among the growth factors studied. Further, we analyzed 23 paraffin-embedded sections of surgically resected gliomas (7 grade II, 8 grade III, and 8 grade IV) by immunohistochemistry. Expression of HGF and Met increased with malignant progression of gliomas, suggesting that gliomas stimulated their invasive proliferation by autocrine HGF production. Neurons and vasculature were HGF-positive, and Met-positive glioma cells gathered around them. The data indicate that neurons and vasculature, which are the main tracks of glioma invasion, augment chemotactic invasion and proliferation of gliomas by paracrine HGF secretion. Clearly HGF plays a critical role in invasive proliferation of glioma cells and it is therefore a candidate target of therapeutic intervention.

Key words: Astrocytoma — HGF — Met — Invasion — Proliferation

Gliomas are the most frequent human brain tumors. Their incidence is reported to be 33% in Japan[1] and even more in the United States[2] of all the primary brain neoplasms. An important biological characteristic of gliomas is their infiltrative proliferation into surrounding normal brain tissue. They invade more distantly and proliferate at a higher rate with progression of their malignant grade.[3–5] Surgical removal is the first-choice treatment for gliomas. However, in order to preserve essential neurological functions, we can not radically resect the marginal zones where normal brain tissues are invaded by glioma cells. Although adjuvant therapies such as radiation and chemotherapy are added to treat tumor remnants after surgery, the five-year actuarial survival rate of glioblastoma is only 5.5 to 7.6%.[1,2] Local tumor recurrence, tumor invasion into neighboring brain tissues, and adverse reaction to adjuvant therapies (e.g., diffuse white matter injury, impairment of intellectual function, brain necrosis, and secondary neoplasms) progressively impair the neurological functions of patients, making their quality of life (Karnofsky rating) very poor. In contrast to other malignant tumors, less than 2% of gliomas metastasize to the extracranial region via blood vessels,[6] so it may be possible to improve the prognosis and quality of life of glioma patients if we can clarify the mechanism of invasive proliferation of glioma.

Invasion and proliferation of gliomas have been studied mainly from the standpoint of growth factors and their receptors, proteases, extracellular matrices, adhesion molecules (for reviews, see refs. 7 and 8), and the cytoskeleton.[9] Cell motility is an important factor for invasion. Various cytokines have been reported to modulate the motility of glioma cells. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β) are representative ones.[10,11] Recently, hepatocyte growth factor (HGF), autocrine motility factor, autotaxin, and macrophage stimulating protein have also been found to promote cell motility.[12–18] Among these candidate motility factors including their receptors, we found that Met, the receptor for HGF, was expressed in all the glioma cell lines tested.

HGF, which was molecularly cloned in 1989,[19] is a multifunctional cytokine. It functions as an autocrine and/or paracrine growth factor in normal situations.[12–14,20] Scatter factor, which was first described as a fibroblast-derived motility factor for epithelial cells, was identical to HGF. HGF and Met are reported to be expressed in gliomas,[21–24] but little is known about their actual role or clinical significance. In this study, we tested the expression of HGF and its receptor Met in four

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glioma cell lines and seven primary cultures. *In vitro* biological activities of HGF in relation to the growth and motility of glioma cells were compared with those of other growth factors, i.e., EGF, bFGF, PDGF-BB, and TGF-β1. Immunohistochemical study of 23 surgical specimens indicated a critical role of HGF in invasive proliferation of gliomas.

MATERIALS AND METHODS

Glioma cell lines Human glioma cell lines A172 and T98G were obtained from JCRB. U87MG and U373MG were obtained from ATCC. These four human glioma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 m*M* L-glutamine, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin (CM10).

Primary culture Seven surgical specimens (two anaplastic astrocytomas and five glioblastomas classified and graded according to WHO standards) were used for the primary culture. No patients had received chemotherapy or radiation therapy prior to surgery. For primary culture, fragments of the surgically resected tumors were rinsed repeatedly with DMEM, minced into 2 mm pieces and digested with 2 mg/ml collagenase type I (Sigma Chemical Co., St. Louis, MO) in DMEM at 37°C for 2 h. The dispersed cells were cultured in DMEM supplemented with 15% FBS. All primary culture lines were used at passage 4 to 9. Immunohistochemical analyses were done to confirm that they were positive for glial fibrillary acidic protein (GFAP).

Detection of c-met and HGF mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) Total cellular RNA was isolated by the guanidinium thiocyanate method. RT-PCR was performed as described by Takahashi *et al.*[25] for *c-met* and by Horie *et al.*[26] for HGF. PCR primers that were used to amplify the cDNA sequences were: for *c-met*, 5′ ^{3905}GGT TGC TGA TTT TGG TCA TGC^{3925} 3′ and 5′ ^{4166}TTC GGG TTG TAG GAG TCT TCT^{4146} 3′ (GenBank accession, J02958); for HGF, 5′ ^{976}AGT ACT GTG CAA TTA AAA CAT GCG^{999} 3′ and 5′ ^{1353}TTG TTT GGG ATA AGT TGC CCA^{1333} (GenBank accession, X16323).

Enzyme-linked immunosorbent assay (ELISA) of HGF production A human HGF ELISA kit (IBL, Fujioka) was used to quantify HGF production of glioma cells *in vitro*. Cells were trypsinized, and 1×10^6 cells were plated in a 100-mm culture dish. After overnight culture, the medium was changed to fresh CM10. After 48 h of incubation, conditioned media were harvested for ELISA, and cells were harvested and counted. The media were concentrated 12.5-fold by ultrafiltration with a Centricon 10 (Amicon, Beverly, MA) and subjected to ELISA according to the manufacturer's protocol. Each sample was assayed in duplicate, and the assay was repeated three times.

[^{3}H]Thymidine incorporation assay Cells were plated in DMEM with 5% FBS at 3 to 6×10^3 cells per well of 96-well culture plates. After an overnight culture, the medium was changed to serum-free DMEM (CM0). Twenty-four hours later, the medium was changed to CM0 containing various growth factors (i.e., tissue culture grade EGF (Sigma Chemical Co.), human recombinant bFGF (Sigma Chemical Co.), human recombinant PDGF-BB (Sigma Chemical Co.), human recombinant TGF-β1 (KBI, Kakogawa), and human recombinant HGF (Sigma Chemical Co.)). After 4 h of incubation, 1 μCi of [^{3}H]thymidine (Amersham, Buckinghamshire, UK) was added, and cells were incubated for 20 h. The cells were then washed with phosphate-buffered saline (PBS), immersed in 100 μl of 10% trichloroacetic acid, placed on ice for 15 min, washed with 10% trichloroacetic acid, solubilized with 100 μl of 0.2 *N* NaOH containing 0.1% sodium dodecyl sulfate, and incubated at 37°C for 30 min. Cellular DNA with [^{3}H]thymidine incorporation was harvested by use of a Combi Cell Harvester (Skatron Instruments, Lier, Norway) and radioactivity was measured with a liquid scintillation counter.

Boyden chamber assay In order to analyze the chemotactic and chemokinetic effects of various growth factors, 24-well cell culture inserts incorporating polycarbonate membranes with 8 μm pore size (#3097, Becton Dickinson Labware, Franklin Lakes, NJ) were used. First, 700 μl of CM0 was put into wells of 24-well plates (lower compartment), and a culture insert was placed in each well. Cells were then trypsinized, and 4 to 8×10^4 cells were suspended in 200 μl of CM10 and placed in the culture insert (upper compartment). After incubation on ice for 30 min followed by 2 h incubation at 37°C for cell attachment, the medium of each compartment was changed to CM10 containing various concentrations of growth factors. For neutralization assay, CM10 containing HGF and anti-HGF monoclonal antibody 24612.111 (IgG1, R & D Systems, Minneapolis, MN) (aHGF mAb) or control normal mouse IgG1 (Vector Laboratories, Inc., Burlingame, CA) (control Ig) was pre-incubated at room temperature for 1 h. The cells were incubated for 5 h at 37°C, fixed with 3% glutaraldehyde in PBS and stained with hematoxylin. Cells on the upper surface of the culture insert membrane were removed by wiping with a cotton swab, and cells that had migrated to the lower side of the membrane were counted with a phase-contrast microscope at 200× magnification. Five fields were counted for each assay. Experiments with duplicate determinations were repeated three times.

Scattering and concentric circle assay Cells were trypsinized, and 6×10^3 cells were resuspended in 6 μl of

CM10 and placed at the center of each well of 12-well culture plates. Cells were incubated at 37°C for an hour and 6 μl of CM10 was added. An hour later, 20 μl of CM10 was added, and the cells were incubated for 2 h. Then 2 ml of CM0 was added to each well. After 24 h (day 0), cells scattered around the margin of the concentric circles were swept off under a phase-contrast microscope to define clearly the margins of the concentric circles. The medium was then changed to that containing various growth factors and this was replaced with fresh medium every third day. Photos were taken on days 0, 1, 2 and 4 for scattering assay. At day nine, cells were fixed with 3% glutaraldehyde in PBS and stained with hematoxylin. The diameter of the concentric circle was measured with slide calipers and the area of the circle was calculated.

Immunohistochemistry Twenty-three formalin-fixed, paraffin-embedded astrocytoma sections (7 WHO grade II astrocytomas, 8 grade III, and 8 grade IV) were used in this study. To enhance the immunoreactivities, autoclave pretreatment of sections was performed according to the method of Shin *et al.*[27] To enhance membrane permeability for aMet and aHGF Abs (IBL, Fujioka), sections were treated with 0.05% saponin in distilled water for 30 min at room temperature. No pretreatment was carried out for staining with anti-neurofilament (MBL, Nagoya) or aGFAP (Amersham) Abs. Chromatographically purified rabbit IgG (Zymed Laboratories, Inc., San Francisco, CA) was used as a negative control. Immunohistochemical detection was carried out using the labeled streptoavidin-biotin method (LSAB kit, Dako Corp., Carpinteria, CA) according to the manufacturer's protocol. Incubation of sections with 2 μg/ml aHGF and aMet Abs was carried out at 4°C for 12 h. Diaminobenzidine (Sigma Chemical Co.) (0.5 mg/ml) was used as the peroxidase substrate, followed by hematoxylin counterstaining.

Immunoreactivity of each cell was determined by comparison with negative control staining with rabbit IgG. All slides were reviewed by three investigators. Three fields at the center of the tumor were selected, and the number of positive cells per 450 tumor cells (150 cells/field, magnification $\times$400) in each case was counted.

Statistical analysis Data were analyzed by use of the two-sample t test.

RESULTS

Expression of c-*met* and HGF in glioma lines Expression of c-*met* and HGF mRNA was examined by RT-PCR. Every cell line expressed c-*met* (Fig. 1A). All primary culture lines expressed HGF mRNA (Fig. 1B). In contrast, only one cell line (U87MG) out of four expressed HGF mRNA. Since this RT-PCR analysis was a qualitative assay to detect the mRNA expression level, we confirmed the HGF production from glioma cells by ELISA. As shown in Fig. 1C, all primary cultured cells produced HGF, while only U87MG out of four cell lines

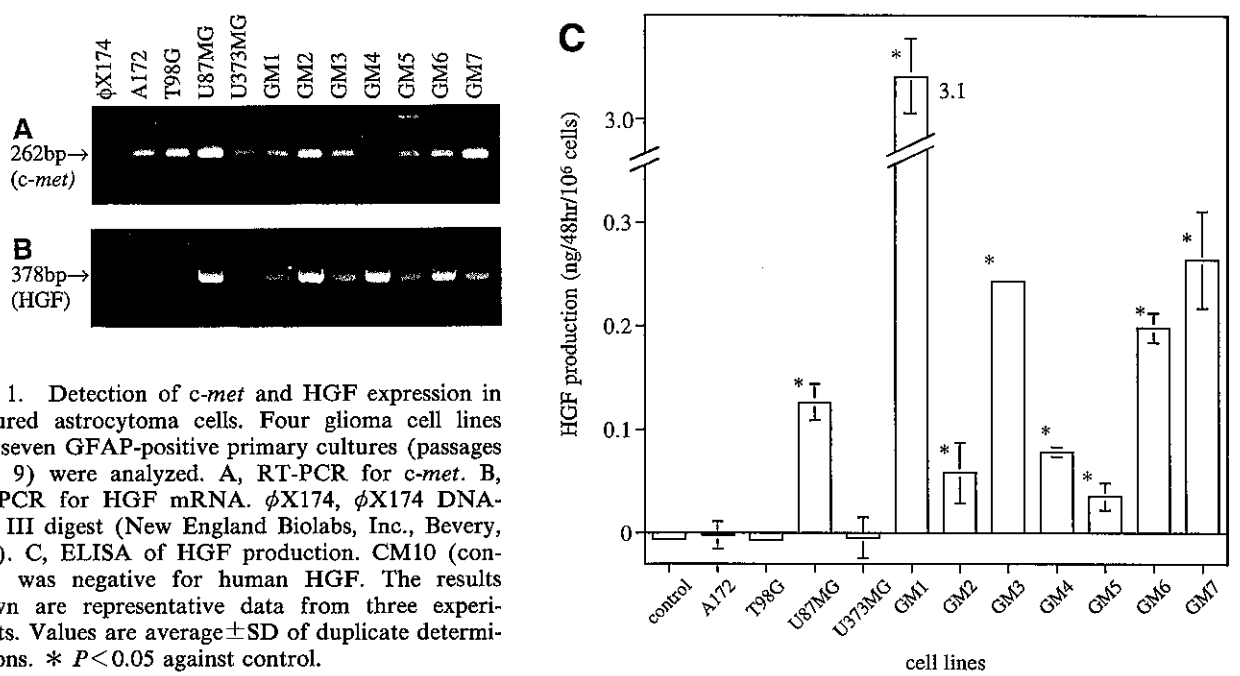


Fig. 1. Detection of c-*met* and HGF expression in cultured astrocytoma cells. Four glioma cell lines and seven GFAP-positive primary cultures (passages 4 to 9) were analyzed. A, RT-PCR for c-*met*. B, RT-PCR for HGF mRNA. ϕX174, ϕX174 DNA-*Hae* III digest (New England Biolabs, Inc., Bevery, MA). C, ELISA of HGF production. CM10 (control) was negative for human HGF. The results shown are representative data from three experiments. Values are average$\pm$SD of duplicate determinations. * $P<0.05$ against control.

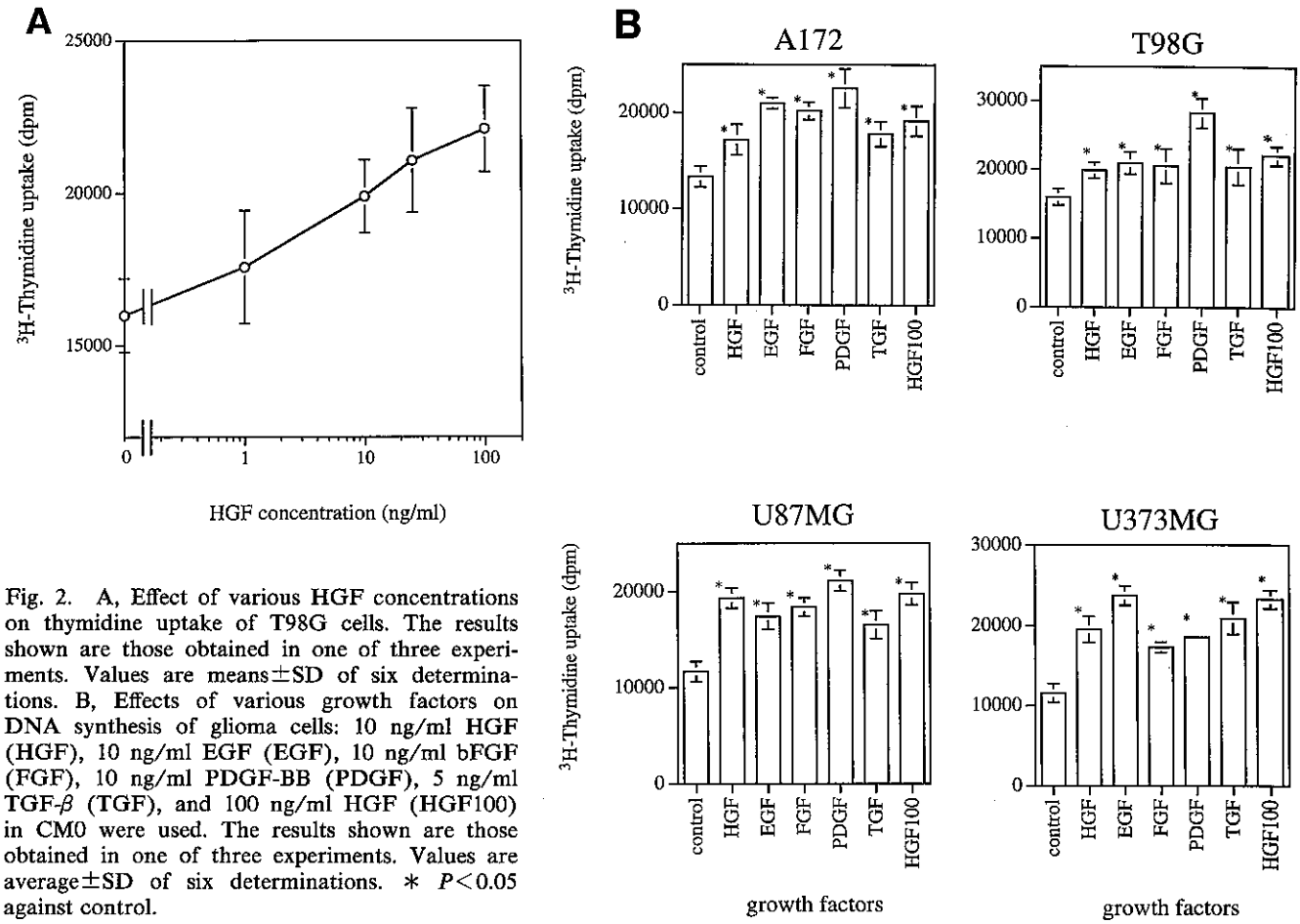


Fig. 2. A, Effect of various HGF concentrations on thymidine uptake of T98G cells. The results shown are those obtained in one of three experiments. Values are means±SD of six determinations. B, Effects of various growth factors on DNA synthesis of glioma cells: 10 ng/ml HGF (HGF), 10 ng/ml EGF (EGF), 10 ng/ml bFGF (FGF), 10 ng/ml PDGF-BB (PDGF), 5 ng/ml TGF-β (TGF), and 100 ng/ml HGF (HGF100) in CM0 were used. The results shown are those obtained in one of three experiments. Values are average±SD of six determinations. * $P<0.05$ against control.

produced HGF. These results were in accord with the results of RT-PCR (Fig. 1B).

[^{3}H]Thymidine incorporation assay HGF in CM0 stimulated DNA synthesis of T98G cells in a dose-dependent manner (Fig. 2A). Maximum DNA synthesis was obtained with 100 ng/ml HGF (1.18 n*M*). A172, U87MG, and U373MG cells also responded to HGF in a similar dose-response relationship (data not shown). Our preliminary study revealed that 10 ng/ml EGF (1.61 n*M*), 10 ng/ml bFGF (610 p*M*), 10 ng/ml PDGF-BB (407 p*M*), and 5 ng/ml TGF-β1 (200 p*M*) were the optimum concentrations to stimulate DNA synthesis of glioma cell lines. Fig. 2B shows the effects on DNA synthesis of various growth factors, each at optimum concentration. HGF was also tested at 10 ng/ml, as this concentration was used to compare the effects of those growth factors on cellular motility. All of these growth factors stimulated DNA synthesis of every glioma cell line.

Cell motility assay Fig. 3A shows the results of checker board assay of T98G and U373MG cells. This experiment discriminates between chemokinetic and chemotactic activity of HGF. When HGF was added to the upper compartment, the number of T98G cells in the lower compartment increased in proportion to the HGF concentration. This indicates that HGF stimulates chemokinetic activity. HGF in the lower compartment increased the number of migrated T98G cells more than the same concentration of HGF in the upper compartment. The result indicates that HGF also stimulates chemotactic activity on T98G cells. In contrast, the number of migrated U373MG cells decreased in relation to the HGF concentration when HGF was added to the upper compartment. The number of migrated U373MG cells increased when HGF was added to the lower compartment. When the same concentration of HGF was added to the upper compartment, migrated U373MG cells decreased rather than increased. The results indicate that HGF acted as a strong chemoattractant for U373MG cells. Thus, we concluded that HGF has chemokinetic and strong chemotactic effects on glioma cells.

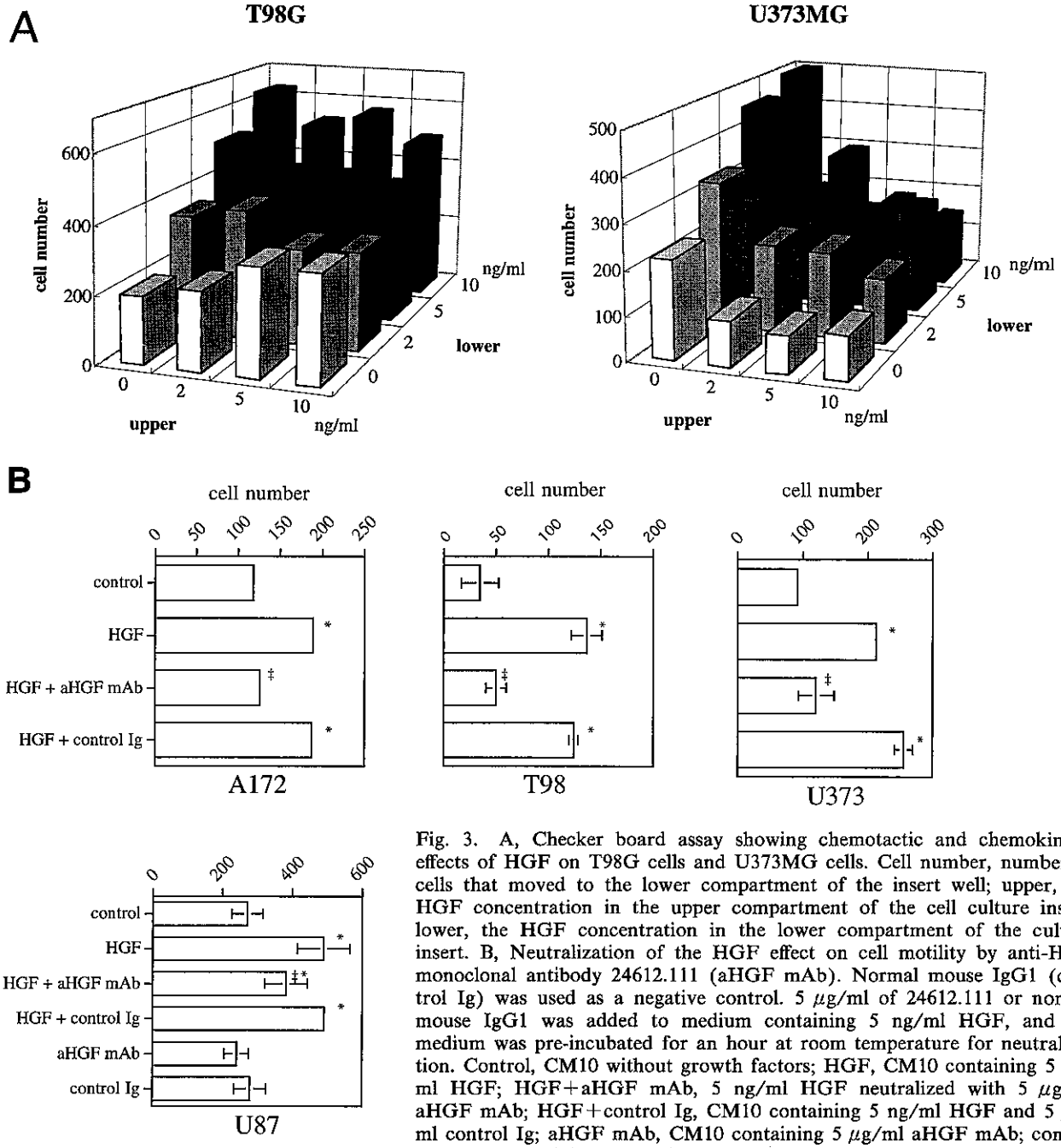


Fig. 3. A, Checker board assay showing chemotactic and chemokinetic effects of HGF on T98G cells and U373MG cells. Cell number, number of cells that moved to the lower compartment of the insert well; upper, the HGF concentration in the upper compartment of the cell culture insert; lower, the HGF concentration in the lower compartment of the culture insert. B, Neutralization of the HGF effect on cell motility by anti-HGF monoclonal antibody 24612.111 (aHGF mAb). Normal mouse IgG1 (control Ig) was used as a negative control. 5 μg/ml of 24612.111 or normal mouse IgG1 was added to medium containing 5 ng/ml HGF, and the medium was pre-incubated for an hour at room temperature for neutralization. Control, CM10 without growth factors; HGF, CM10 containing 5 ng/ml HGF; HGF+aHGF mAb, 5 ng/ml HGF neutralized with 5 μg/ml aHGF mAb; HGF+control Ig, CM10 containing 5 ng/ml HGF and 5 μg/ml control Ig; aHGF mAb, CM10 containing 5 μg/ml aHGF mAb; control Ig, CM10 containing 5 μg/ml mouse IgG1. Experiments with duplicate determination were repeated three times. Values are average$\pm$SD of three independent experiments. $*$ $P<0.05$ against control; $\ddagger$ $P<0.05$ against HGF.

Neutralization assay shows that the effects of HGF on the motility of A172, T98G, and U373MG cells were efficiently blocked by anti-human HGF (Fig. 3B). The effect of HGF on the motility of U87MG cells was also blocked with 5 μg/ml of the aHGF mAb, albeit incompletely. Since U87MG cells produced HGF by themselves (Fig. 1, B and C), the autocrine HGF activity was not efficiently blocked by 5 μg/ml of the aHGF mAb.

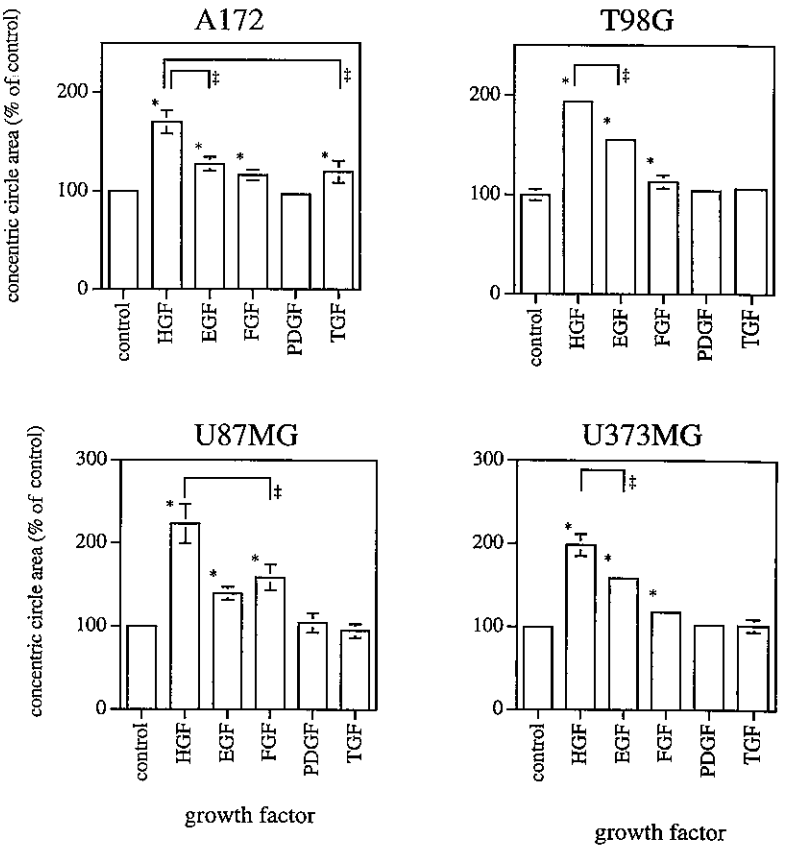


Fig. 4. Effects of various growth factors on cell kinetics in Boyden chamber assay. Growth factors (10 ng/ml) were added to either the upper (U) or the lower (L) compartment of culture inserts to compare chemokinetic and chemotactic functions. Values are average±SD from four independent experiments with duplicate determinations. * $P<0.05$ against control; ‡ $P<0.05$ against HGF.

The basal motility of U87MG was higher than that of the other cell lines, presumably because of this autocrine HGF.

The effects of various growth factors upon cell motility are shown in Fig. 4. HGF affected the motility of all four cell lines tested. EGF induced both chemokinetic and chemotactic action. EGF affected three out of four cell lines (A172, T98G, U87MG). PDGF-BB acted as a strong chemoattractant. PDGF-BB affected two out of four cell lines (A172 and T98G). In contrast, bFGF stimulated cell motility mainly in a chemokinetic manner. bFGF affected two out of four cell lines (T98G and U87MG).

Scattering assay Fig. 5 shows the scattering effect of HGF. The effects of 5 ng/ml HGF were neutralized by 5 μg/ml of the aHGF Ab in U373MG cells. The effect of autocrine HGF was blocked with 25 μg/ml of aHGF Ab. Fig. 6 shows the effects of various growth factors on cell scattering. U373MG cells were scattered by HGF and EGF. bFGF and PDGF-BB did not affect scattering of this cell line. TGF-β1 inhibited scattering of U373MG cells. Similar results were obtained in A172, T98G, and U87MG cells, with the one exception that bFGF moderately stimulated scattering of A172 cells (data not shown).

Concentric circle assay We examined two-dimensional expansion of the tumor in a concentric circular fashion, which results from both cell motility and cell proliferation. In this assay, the concentration of HGF in the medium is equilibrated (i.e., there is no gradient of HGF concentration during the 9-day culture). Thus, scattering and chemokinetic actions are the predominant factors for cell motility. HGF had the strongest effect among the growth factors in all the four cell lines. Although PDGF-BB stimulated proliferation of glioma cells, the proliferated cells piled up without spreading, probably because its effect on scattering and chemokinesis, but not chemotaxis, was weak (Figs. 6 and 7).

Immunohistochemistry Expression of HGF was studied using 23 surgical specimens. Five out of seven grade II astrocytoma sections were weakly positive with HGF (12 to 29%), and two were moderately positive (38 and 51%). In eight grade III astrocytomas, five were moderately positive (37 to 65%), and three were strongly positive. In eight grade IV astrocytomas, one was moderately positive with HGF (50%) and seven were strongly

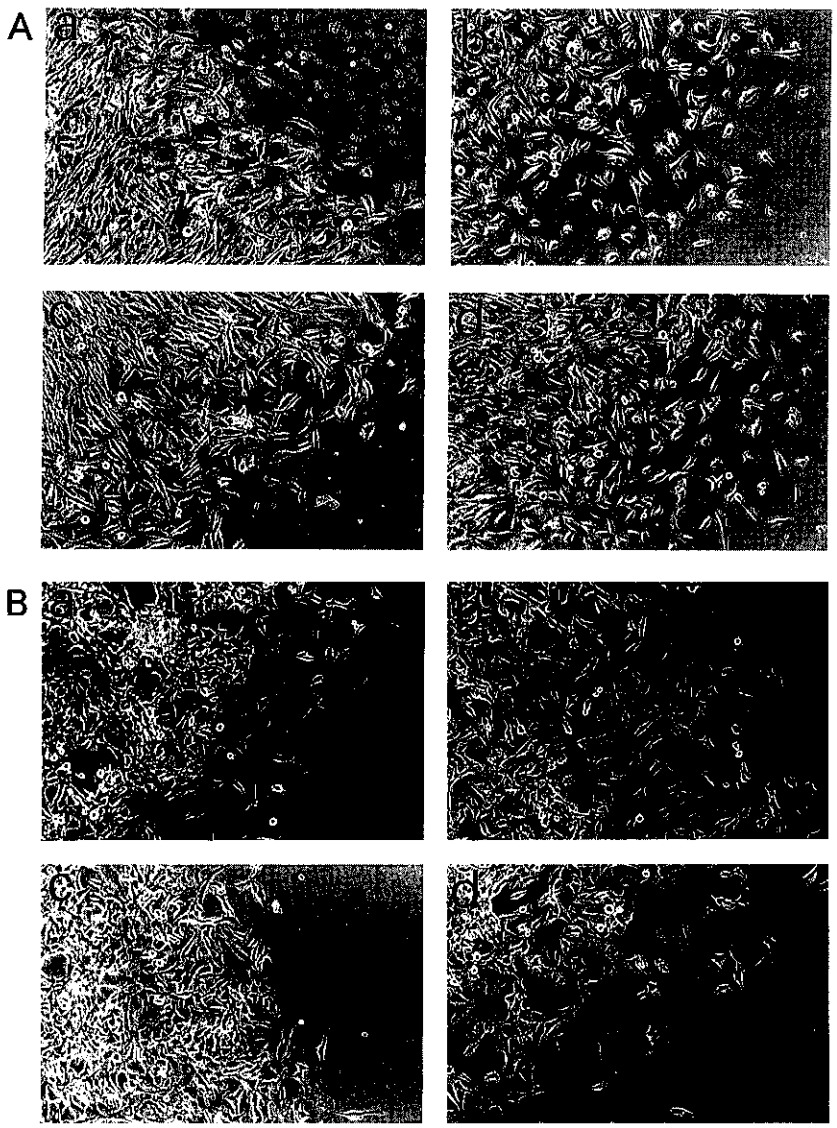


Fig. 5. Neutralization of HGF effects on cell scattering with aHGF mAb. Normal mouse IgG1 was used as a negative control. A, Scattering of U373MG cells. (a) CM10 (control). (b) CM10 containing 5 ng/ml HGF. (c) CM10 containing 5 ng/ml HGF neutralized with 5 μg/ml aHGF mAb. (d) CM10 containing 5 ng/ml HGF and 5 μg/ml control Ig. B, Scattering of U87MG cells. (a) CM10 (control). (b) CM10 containing 5 ng/ml HGF. (c) CM10 containing 25 μg/ml aHGF mAb. (d) CM10 containing 25 μg/ml control Ig.

positive (>90%). GM1, a grade IV astrocytoma which produced abundant HGF *in vitro* (Fig. 1, B and C), demonstrated strongly positive immunoreactivity for HGF (100%). We also examined expression of Met in surgical specimens. In seven grade II astrocytomas, five were weakly positive (9 to 24%) and two were moderately positive (34 and 36%). In eight grade III astrocytomas, immunoreactivity was moderate in seven (40 to 70%), and strong in one (100%). In eight grade IV astrocytomas, immunoreactivity was weak in one (11%), and strong in seven (>77%). The sections stained with control rabbit IgG showed no immunoreactivity. These results indicate that expression of HGF and its receptor Met increased according to the grade of malignancy (Table I and Fig. 8).

Neurons in normal brain and vascular endothelial cells were positively stained by both aHGF and aMet Abs. Smooth muscle cells in the vasculature were also HGF-positive. GFAP-positive cells gathered around the vasculature (Fig. 9A) and around cells with a neuron-like morphology (Fig. 9B). Those neuron-like cells were confirmed to be positively stained by anti-neurofilament antibody (Fig. 9C). Neurons and vasculature, which were surrounded by GFAP-positive cells, were HGF-positive (Fig. 9D). The GFAP-positive cells around neurons and vasculature were positively stained with aMet Ab (Fig. 9E). These features were observed in all the eleven surgical specimens which contained marginal zones of glioma invasion (five in grade II astrocytoma, four in grade III astrocytoma, and two in grade IV astrocy-

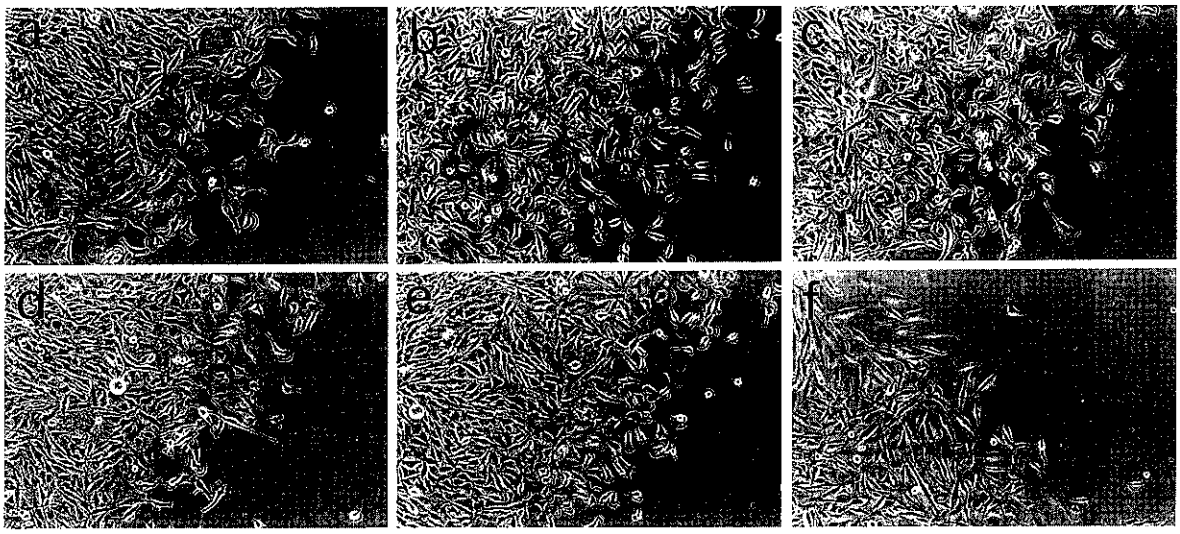


Fig. 6. Scattering effect of various growth factors on U373MG cells. (a) CM10 (control). (b) CM10 with 10 ng/ml HGF. (c) CM10 with 10 ng/ml EGF. (d) CM10 with 10 ng/ml bFGF. (e) CM10 with 10 ng/ml PDGF-BB. (f) CM10 with 5 ng/ml TGF-β1.

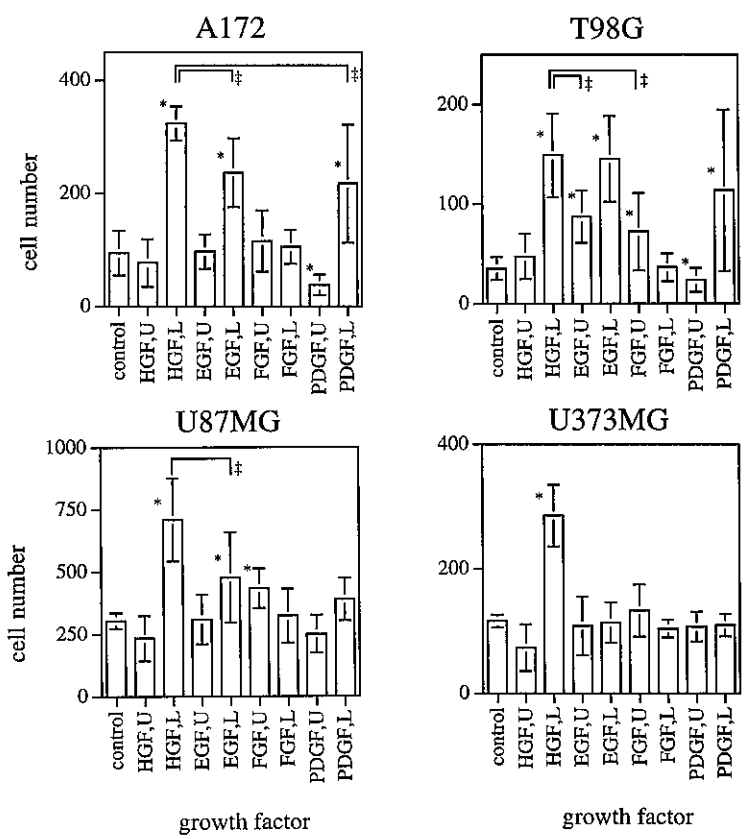


Fig. 7. Concentric circle assay. Cells were cultured for 9 days in the presence of various growth factors. The area was expressed as % of the control. HGF, 10 ng/ml HGF; EGF, 10 ng/ml EGF; FGF, 10 ng/ml bFGF; PDGF, 10 ng/ml PDGF-BB; TGF, 5 ng/ml TGF-β1. Values are average$\pm$SD from two independent experiments with duplicate determinations. * $P<0.05$ against control; ‡ $P<0.05$ against HGF.

Table I. HGF and Met Expression in Astrocytomas: Immunohistochemical Analysis of 23 Clinical Samples

Case No.[a]	Grade[b]	Age/Sex	Location[c]	Immunostaining[d]	
				HGF	Met
1	II	40/M	T	+	++
2	II	33/F	T	++	+
3	II	51/F	F	+	+
4	II	30/F	F	+	+
5	II	41/M	T	+	++
6	II	22/M	F	+	+
7	II	23/M	F	++	+
8 (GM3)	III	59/M	F	++	++
9	III	39/M	T	+++	++
10	III	22/M	F-T	+++	++
11	III	60/M	F	++	++
12	III	45/F	F	++	++
13	III	68/F	T	++	++
14	III	54/F	T	++	++
15	III	14/M	IV	+++	+++
16 (GM1)	IV	72/M	T-P	+++	+++
17 (GM2)	IV	60/F	F	+++	+++
18 (GM4)	IV	48/M	T-P	+++	+++
19	IV	65/M	P	+++	+++
20	IV	54/F	O	++	+
21	IV	46/F	F	+++	+++
22	IV	46/F	T	+++	+++
23	IV	50/F	F	+++	+++

a) Primary cultures of Cases 8, 16, 17, and 18 were also used in RT-PCR.
b) Grading according to WHO standards.
c) F, frontal lobe; T, temporal lobe; P, parietal lobe; O, occipital lobe; F-T, fronto-parietal; T-P, temporo-parietal; IV, intraventricular.
d) −, negative (<5%); +, weakly positive (5–30%); ++, moderately positive (30–70%); +++, strongly positive (70% <). The table shows representative data of three investigators.

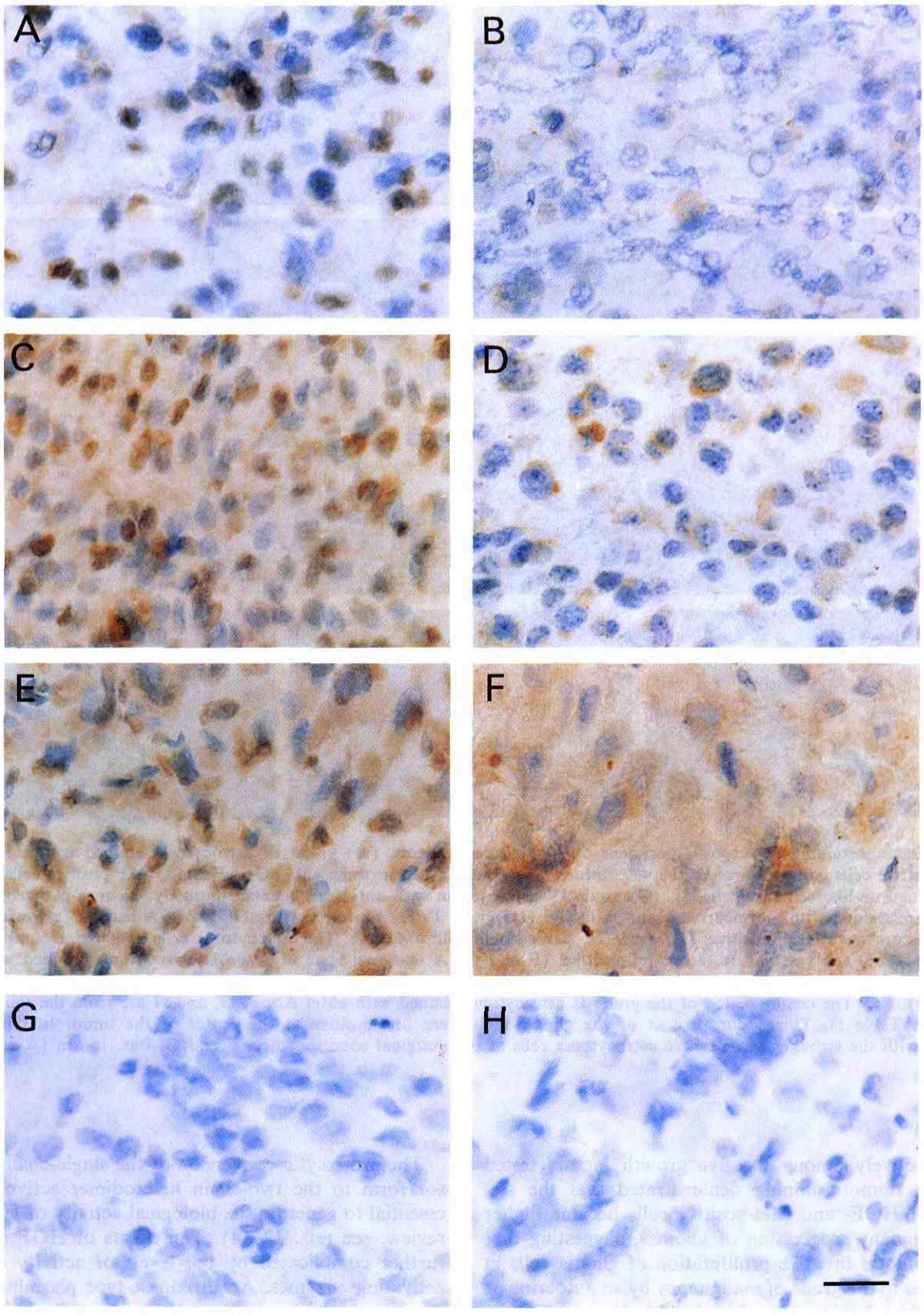
toma). Interestingly, glioma cells that gathered around the vasculature and neurons at the marginal zones were strongly positive for Met, even in grade II astrocytomas, which were weakly positive for Met at the center of the tumor (Fig. 9, F, G and H).

DISCUSSION

HGF and its receptor Met are expressed in normal brain,[28–31] reactive astrocytes,[32] and gliomas (Fig. 1 and Table I).[21–24] In normal brain, HGF mRNA is localized in neurons, primarily in the hippocampus, the amygdala, and the cortex. It was also present at high levels in ependymal cells and the choroid plexus. c-*met* mRNA was expressed in neurons, preferentially in the CA-1 area of the hippocampus, the cortex, neurons of the olfactory bulb, and the septum.[30, 31] Reactive astrocytes in diseased brain also express Met (data not shown) and an increased level of HGF,[32] indicating that non-tumorous astrocytes have the potential to express HGF and Met in response to pathological changes in the brain. In such situations, some stimulation induces expression of HGF and Met in astrocytes, facilitating migration or proliferation. HGF and Met are also found in gliomas.[21–24] In our experiments, cells of all seven primary cultures, whose passage number was less than ten, were HGF-positive (Fig. 1, B and C). In contrast, three out of four glioma cell lines in long-term *in vitro* culture were HGF-negative (Fig. 1, B and C). One of our primary culture lines with a passage number of over 20 was also HGF-negative (data not shown). It is likely that some cell lines lose their HGF expression during long-term culture. Since other cytokines are abundant in FBS and stimulate proliferation of glioma cells, HGF production is unnecessary for glioma cells to proliferate *in vitro*. Therefore, the frequency and/or quantity of HGF synthesis may gradually decrease during long-term passage in culture. Since Met expression offers no advantage for the growth of glioma cells *in vitro*, glioma cells may also lose Met expression during long-term culture *in vitro*. Wullich *et al.*[22] reported a decrease in c-*met* gene amplification in glioma cells during cell culture.

HGF has neurotrophic activity on central nervous system neurons,[31, 33] but the clinical significance of HGF in gliomas has been obscure. During the preparation of this manuscript, two reports concerning the role of HGF in glioma cells have been published.[34, 35] Moriyama *et al.*[34] have reported that HGF stimulates the motility of glioma cells. Rosen *et al.*[35] reported that expression of HGF and Met correlated with the grade of malignancy. In this report, we have shown that HGF promotes motility and proliferation of glioma cells *in vitro*. [^{3}H]Thymidine incorporation assay disclosed that HGF stimulated proliferation of glioma cells in a dose-dependent manner (Fig. 2). Scattering assay and Boyden chamber assay demonstrated that HGF induced both chemokinetic and strong chemotactic activities of glioma cells (Figs. 3–6). Since HGF acts as a chemoattractant, glioma cells infiltrate upstream in a gradient of HGF concentration. This may be advantageous for both proliferation and expansion of glioma cells. Concentric circle assay revealed that HGF stimulated expansive proliferation of glioma cells

Fig. 8. Immunostaining of astrocytoma paraffin sections. (A) grade II astrocytoma with aHGF Ab. (B) grade II astrocytoma with aMet Ab. (C) grade III astrocytoma with aHGF Ab. (D) grade III astrocytoma with aMet Ab. (E) grade IV astrocytoma with aHGF Ab. (F) grade IV astrocytoma with aHGF Ab. (G) grade IV astrocytoma with rabbit IgG1 (negative control for aHGF immunostaining). (H) grade IV astrocytoma with rabbit IgG1 (negative control for aMet immunostaining). Bar: 15 μm.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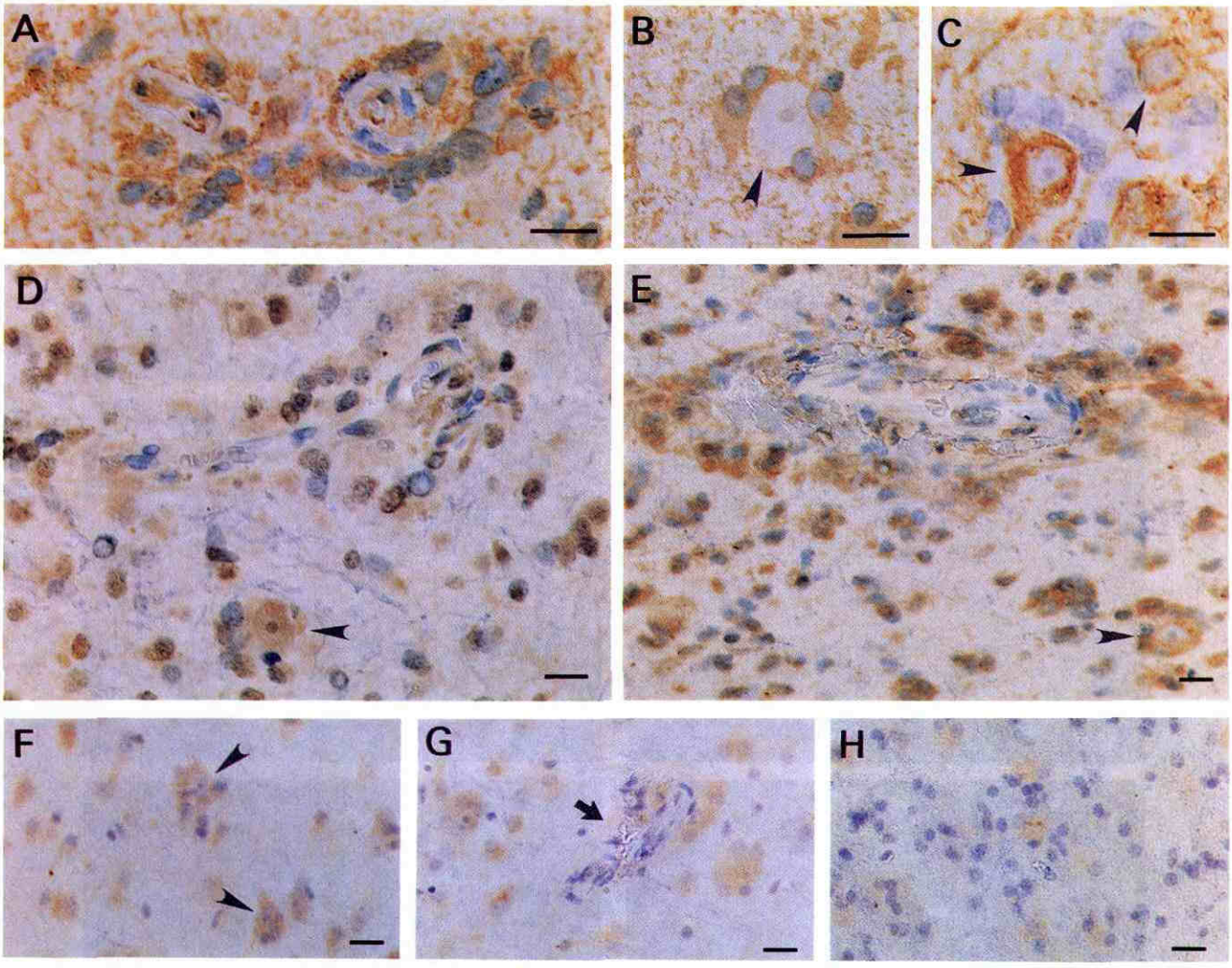

Fig. 9. Immunostaining of surgical specimens of astrocytoma patients. (A) grade IV astrocytoma stained with aGFAP Ab. GFAP-positive cells gathered around the vasculature. (B) grade IV astrocytoma with aGFAP Ab. GFAP-positive cells gathered around a neuron-like cell (arrowhead). (C) grade IV astrocytoma with anti-neurofilament antibody. Neuron-like cells (arrowheads) were stained with anti-neurofilament antibody. (D) grade IV astrocytoma with aHGF Ab. A neuron (arrowhead) and the vasculature were HGF-positive. (E) grade IV astrocytoma with aMet Ab. The astrocytoma cells gathered around a neuron (arrowhead) and the vasculature were Met-positive. (F and G) A marginal zone of grade II astrocytoma, stained with aMet Ab. Strongly Met-positive astrocytoma cells at the marginal zone gathered around neurons (F; arrowheads) and the vasculature (G; arrow). (H) The center region of the grade II astrocytoma stained with aMet Ab. F, G, and H are from the same patient (case 5 in Table I). Thirty-four percent of the glioma cells were Met-positive at the center of the tumor in this patient. Compare with the strongly Met-positive astrocytoma cells at the marginal zone shown in F and G. Bar: 15 μm (A–E), 30 μm (F–H).

most effectively among the five growth factors tested (Fig. 7). Immunostaining demonstrated that the frequency of HGF- and Met-positive cells became higher with malignant progression of gliomas, suggesting that HGF promotes invasive proliferation of glioma cells in proportion to the grade of malignancy by an autocrine or paracrine mechanism (Fig. 8 and Table I).

The proteolytic conversion of the single-chain precursor form to the two-chain heterodimer active form is essential to generate the biological activity of HGF (for review, see refs. 12–14). The effects of HGF could be further complicated by the levels of activity of HGF activating enzymes. An urokinase-type plasminogen activator, a kind of serine proteinase, was supposed to be an

HGF activator (for review, see refs. 12–14). Expression of this enzyme correlates with malignant progression of gliomas.[36, 37] Recently, a specific HGF activator, which also belongs to the serine proteinase family, was cloned.[38] Its presence in glioma tissues was confirmed by Moriyama *et al.*[24] by RT-PCR of five surgical specimens. These data suggest that glioma cells activate autocrine HGF to stimulate themselves. Even if they lack these HGF-activating enzymes, Met-positive glioma cells are stimulated by the active-form HGF from neighboring tissues (e.g., neurons and vasculature) in a paracrine fashion and infiltrate into the surrounding structures.

Extracellular matrices are also important factors for cellular proliferation and invasion. Tumor cells in the brain are embedded in a proteoglycan-rich environment with a high water content.[8] The region is physically weak, and glioma cells easily infiltrate it. Heparan sulfate, the proteoglycan, known as a low-affinity receptor for HGF,[39] is also present on the cell surface of brain tissues and gliomas.[40, 41] Heparan sulfate works as a reservoir for storage of HGF in the brain, and generates a gradient of HGF concentration, which allows the chemotactic activity of HGF to be manifested.

There are several pathways for glioma cells to invade the brain; local infiltration, white matter tracts (myelin pathways), and spreading along ependyma and through the cerebrospinal fluid. Local infiltration includes spreading along neurons and blood vessels.[42, 43] HGF is produced by neurons, endothelial cells, and smooth muscle cells in blood vessels (Fig. 9).[20] The secreted HGF is stored in the heparan sulfate around these cells and attracts glioma cells through its chemotactic activity. Glioma cells invade along these pathways and gather around HGF-producing cells, as shown in Fig. 9. Microglias also produce HGF,[29, 33] which helps migration of glioma cells via white matter tracts. Autocrine secretion of HGF from glioma cells is also an important factor. Autocrine HGF with its strong growth-stimulating and chemokinetic activities promotes glioma cell invasion into surrounding structures in random directions along mechanically weak regions, such as white matter tracts. Since HGF is also produced by ependymal cells and the choroid plexus, it may further promote the glioma cells' invasive proliferation along the ependyma and through the cerebrospinal fluid.

HGF production may not always facilitate invasion of glioma cells. When the chemotactic effect of HGF is strong, as in U373MG cells (Fig. 3A), migration of glioma cells may be restricted because of the relatively lower HGF concentration in surrounding structure tissues compared to the glioma mass which produces HGF. Under such circumstances, the partial removal of a tumor for mass reduction results in a relative increase of HGF concentration in surrounding structures, and promotes infiltration of residual glioma cells.

Tumor angiogenesis supports enlargement of solid tumors and the metastasis of cancer cells. Gliomas are hypervascularized tumors. PDGF, bFGF, VEGF, interleukin 8, and TNF-α are produced by gliomas and promote neoangiogenesis and endothelial proliferation in high-grade gliomas.[44–46] As Nagano *et al.*[43] reported, glioma cells infiltrated toward the vasculature. This phenomenon may be caused by chemotactic activity of HGF as indicated by our motility assay and immunostaining results. Our findings suggest a critical role of HGF in the interaction between infiltrative growth and neoangiogenesis of gliomas. Specifically, blood vessels attract glioma cells by chemotactic activity of HGF, and the glioma cells infiltrating toward the vasculature produce cytokines (e.g., PDGF, bFGF, VEGF) that promote angiogenesis and endothelial proliferation. HGF is also a potent angiogenesis factor.[47, 48] The increase in HGF production from newly constructed blood vessels further attracts glioma cells toward the vasculature. This chain reaction leads to a vicious cycle of invasive growth and neoangiogenesis.

In conclusion, HGF strongly promotes motility and proliferation of glioma cells, probably more commonly and effectively than the other growth factors, and it is also involved in invasive expansion and neoangiogenesis of gliomas. EGF, PDGF, bFGF are believed to be essential for malignant progression of astrocytomas, and their receptors increase with the grade of malignancy.[49–51] Possible cooperation of these growth factors with HGF remains to be studied. We conclude that HGF plays a critical role in the invasive proliferation of glioma cells and it appears to be a promising target for therapeutic intervention.

(Received January 29, 1997/Accepted March 21, 1997)

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