Met and Hepatocyte Growth Factor/Scatter Factor Expression in Human Gliomas

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ABSTRACT

Using double immunofluorescence staining and quantitative confocal laser scan microscopy, we show that the intensity of hepatocyte growth factor/scatter factor (HGF/SF) and Met staining in human primary brain tumors increases with the grade of malignancy and is prevalent in both the infiltrating tumor cells and endothelial hyperplastic areas. HGF/SF and Met are also expressed in vitro in glioblastoma multiforme cell lines as well as in normal human astrocyte (NHA) cells. Moreover, HGF/SF stimulates tyrosine phosphorylation of Met in both glioma cell lines and NHA cells, but only the glioma cell lines proliferate and become motile and invasive in response to HGF/SF, whereas the NHA cells are nonresponsive. These results implicate autocrine/paracrine Met-HGF/SF signaling in glioma tumorigenesis and suggest that HGF/SF signaling through Met is negatively regulated in NHA cells.

INTRODUCTION

Gliomas are the most frequent and malignant form of human brain tumors (1). These tumors are highly invasive, irrespective of their histological grade of malignancy. Even low-grade tumors can be poorly demarcated and are rarely encapsulated. Consequently, surgical removal is complex, the tumors recur frequently, and the 2- and 5-year survival rates for malignant glioma are less than 15 and 5%, respectively (1). The factors and mechanisms involved in tumor invasion and proliferation are poorly understood. With human gliomas as well as other cancers, several growth factors, including epidermal growth factor, platelet-derived growth factors, fibroblast growth factor, and transforming growth factors α and β and their receptors have been implicated in the cancer phenotype (2).

HGF/SF is a multipotential modulator of biological activities in a variety of cell types (3, 4). Acting through the Met tyrosine kinase receptor, HGF/SF functions as a mitogen (5), stimulates cell motility and invasiveness (5, 6), and participates as a morphogen in mediating lumen formation and branching morphogenesis (7—9). HGF/SF is also a potent angiogenic factor and stimulates endothelial cell proliferation, migration, and morphogenesis (10, 11). We have demonstrated in several rodent and human model systems that Met-HGF/SF signaling induces both tumorigenic and metastatic behavior (4, 6, 12, 13). Met-HGF/SF signaling and Met activation, overexpression, or activation by point mutation have been associated with a variety of human cancers, including brain tumors (4, 11, 14). Yamada et al. (15) detected HGF/SF and Met immunoreactivity in astrocytes and microglia and met mRNA expression in glioma tissues (16). HGF/SF stimulated growth and induced chemotactic activity in glioma cell lines (17), and met gene amplification has been detected in glioblastoma cells and tissues (18). More recently, Rosen et al. (11) have shown that HGF/SF is produced by human glioma cells and brain microvascular endothelial cell lines. Moreover, they found HGF/SF immunoreactivity in 19 of 20 human primary brain tumors. Here we examine the expression of HGF/SF and Met in human primary brain tumors and show that not only are HGF/SF and Met in primary brain tumors, but glioma cell lines are autocrine for Met and HGF/SF signaling, and HGF/SF induces glioma cell proliferation, migration, and invasion in vitro.

MATERIALS AND METHODS

Cell Culture, Antibodies, and Immunocytochemistry. U-373, U-118, U-138, SW-1783, and DBTGR human glioma cell lines and SK-LMS-1, a human leiomyosarcoma cell line, were obtained from American Type Culture Collection (Rockville, MD). Human embryonic lung fibroblast cells (MRC-5) were kindly provided by Dr C. Medicci (University of Parma Medical School, Parma, Italy). NHA (a NHA cell culture derived from fetal brain) was purchased from Clonetics Corp. (San Diego, CA) and propagated in culture medium provided by the manufacturer (AGM-Bulkit). All experiments with NHA cells were performed at passage 5. All other cell lines were grown in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution. Cells were maintained in culture in a standard humidified incubator at 37°C in 5% carbon dioxide. Materials for tissue culture were purchased from Life Technologies, Inc. (Gaithersburg, MD). Growth factor-reduced Matrigel was obtained from Becton Dickinson (Bedford, MA).

Human HGF/SF was purified from the supernatant of transformed NIH 3T3 cells as described previously (13). HGF/SF concentrations are presented as scatter units per milliliter; 5 scatter units is equivalent to approximately 1 ng of protein. Monoclonal antihuman HGF neutralizing antibody (mouse IgG1) was purchased from R&D Systems (Minneapolis, MN).

C-28 is a rabbit polyclonal antibody raised against the COOH-terminal 28 amino acids of the human Met gene product (7, 13). Mouse monoclonal antibody against phosphotyrosine (anti-P-Tyr) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). D1 is a mouse monoclonal antibody (IgG2a) raised against NIH 3T3 cells transfected with human met (pRS2). Mouse monoclonal antibody against actin was purchased from Boehringer Mannheim (Indianapolis, IN).

For immunocytochemistry analysis, cells were grown under standard conditions in DMEM and 10% FBS to a semiconfluence on Labtek slides (Nunc, Naperville, IL). Slides were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min. After extensive washing with PBS, cells were treated with 0.2% Triton X-100 for 10 min and washed again with PBS. To minimize nonspecific binding, chamber slides were incubated with normal sheep serum for 45 min. Slides were then incubated with either D1 antibody (1:50 dilution) or nonimununized mouse serum for 2 h at room temperature. Secondary antibody incubation (FITC-conjugated goat antimouse IgG at 1:100 dilution in PBS) was carried out for 1 h. Chamber slides were then washed and counterstained with DAPI (1%) for 10 min. After the slides were covered with Gel/Mount (Biomeda), the cells were examined by CLSM using a Zeiss CLSM LSM3 microscope (7).

Tumor Materials, Histological Grading, and Immunofluorescence Staining. Tumor specimens were obtained from 32 patients with primary brain tumors from either diagnostic biopsies or therapeutic neurosurgical resection. The biopsy samples were from either the cerebral hemispheres or the cerebellum. Specimens were paraffin-embedded, sectioned at 4 μm, mounted on aminophenyltriethoxysilane-coated slides, and dried overnight. These tumors were diagnosed at Brook General Hospital (London, United Kingdom). Independent confirmation of the histopathological diagnosis in each case was
kindly provided by Drs. Andrew F. Dean and Raskhala Doshi (Department of Neuropathology, Institute of Psychiatry, London, United Kingdom). From one paraffin block, one or two representative slides were selected. Tumors were classified according to WHO nomenclature, and grading ranged from I to IV: low-grade astrocytoma, grade II (n = 6); anaplastic astrocytoma, grade III (n = 11); and glioblastoma multiforme (grade IV; n = 15).

Tumor sections were subjected to double immunofluorescence analyses (7). Sections were permeabilized with 0.2% Triton X-100 in PBS for 10 min and rinsed in D-PBS (Life Technologies, Inc.). Specimens were blocked with 1% BSA in PBS for 30 min, incubated with C-28 anti-Met (1:50 dilution in PBS) for 2 h, and rinsed in D-PBS for 1 h. Sections were then incubated with FITC-conjugated goat anti-rabbit IgG (1:40 dilution in PBS; Boehringer Mannheim) for 1 h. After washing, slides were incubated again with PBS containing 1% BSA for 20 min and exposed to a mouse monoclonal antihuman antibody (7-1; raised against purified single-chain human HGF/SF by the antibody production unit at ABL) for 2 h, rinsed in D-PBS, and incubated with goat antigoat IgG conjugated to rhodamine (1:20 dilution in PBS; Boehringer Mannheim) for 1 h. Slides were thoroughly washed in D-PBS, rinsed in distilled water, and mounted with GelMount. All procedures were carried out at room temperature.

CLSM Imaging and Optimas Quantification Procedure. Fluorescent-stained tumor sections were analyzed using a Zeiss microscope (CLSM 310). The microscope was configured with a 25 mW argon, UV-coherent, internal HeNe laser with the appropriate lines (488, 347, and 543) for FITC, DAPI, and rhodamine excitation. Nomarski images were made using the 543 green laser and appropriate polarizing lenses. Photographs were generated using either a Sony color video printer (UP5200 MD Magiview, Focus Graphics 4700) and 35-mm camera film back (100 ASA Ektachrome) or a Codonics NP600 color printer as described previously (7).

All images were analyzed using the Optimas 5.2 Image Analysis software program. Nomarski images were used as the basis for delineation of proper regions of interest. Masked areas were then drawn over the appropriate areas and saved. The corresponding fluorescent image was recalled, the masked area was overlaid, and the data in the form of pixel counts and the number of pixels at a particular intensity were exported to Microsoft Excel 5.0 for summation and calculation. The mean gray value, Gy, was determined as follows: Gy = 75x (number of pixels × intensity)/75x (number of pixels) (19).

Western Analysis. Semiconfluent cells were grown in DMEM and 10% FBS in 100-mm culture dishes, serum-deprived overnight, washed three times with sterile PBS, and incubated in the presence or absence of HGF/SF (200 units/ml) in fresh culture medium for 1, 2, 4, or 8 h. After incubation, the medium was removed, and the culture plates were washed three times with cold PBS. Cells were lysed in 1 ml of lysis buffer (20 mM Pipes, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, and 1.5 mM MgCl\textsubscript{2} (pH 7.4)) containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) plus SDS at a final concentration of 0.1%. The collected lysate in Eppendorf tubes was placed on ice for 30 min. After centrifugation (20 min; 16,000 × g) at 4°C, supernatants were isolated, and the protein concentration was determined (BCA protein assay; Pierce, Rockford, IL). Ten μg protein from each sample were resolved on a 4–12% polyacrylamide Tris-glycine gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Membranes were blocked with 5% BSA in rinse buffer (1.5 mM NaCl, 200 mM Tris, and 0.1% Tween 20) for 1 h, washed in rinse buffer for 10 min, and then incubated with 1 μg/ml C-28 anti-Met for 1 h at room temperature. Membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1000 dilution; Boehringer Mannheim) for 1 h at room temperature, washed for 30 min, treated with the enhanced chemiluminescence detection system from Amersham (Arlington Heights, IL), and then stripped and reprobed with 1 μg/ml antiactin.

Immunoprecipitation. Cells were grown in DMEM and 10% FBS in 100-mm culture dishes, serum-deprived overnight, and incubated in the presence of absence of HGF/SF (200 units/ml) in DMEM for 0, 15, 30, 60, or 90 min. Treated cells were washed with cold PBS, lysates were prepared, and protein concentration was determined as described for Western analysis. Clarified lysates (0.5 mg of protein) were immunoprecipitated overnight with 1 μg/ml C-28 anti-Met at 4°C. At the end of the incubation period, 50 μl of protein A-agarose (Sigma Chemical Co., St. Louis, MO) at a final concentration of 2% were added to each sample and then incubated for an additional 3 h at 4°C. The samples were then centrifuged and washed with cold lysis buffer (without SDS) three times. The supernatants were removed carefully, and 20 μl of 2X SDS sample buffer (with DTT) were added to each sample. After the samples were boiled for 5 min and centrifuged for 5 min at 16,000 × g, the supernatants were analyzed by SDS-PAGE. For Western analysis with anti-P-Tyr, membranes were incubated with 5 μg/ml primary antibody as described above.

Mitogenic Assays. Cells were seeded into 96-well microtiter plates (Costar, Cambridge, MA) at 5 × 10\textsuperscript{4} cells/well in 200 μl of DMEM containing 10% FBS. After a 24-h incubation at 37°C, plates were washed with PBS, and cells were refed with serum-free medium for 24 h. HGF/SF was then added in DMEM, and cells were incubated for 20 h. Control samples received DMEM alone or DMEM and 10% FBS. After incubation in [\textsuperscript{3}H]thymidine (Amersham) at 5 μCi/well for 4 h, cells were lysed at room temperature with 0.1 ml of 20 mM NaOH and 0.1% SDS for 15 min. The lysates were used for scintillation counting (20).

In Vitro Migration and Invasion Assays. Cell migration assays (16) were performed using 24-well transwell units with 8-μm polycarbonate filters (Costar). The lower compartment of each transwell unit contained 500 μl of DMEM containing 1% BSA and various concentrations of HGF. In several experiments, the lower compartment also contained 1 μg/ml anti-HGF neutralizing monoclonal antibody. Cells were freshly harvested by trypsinization and counted, and 10\textsuperscript{4} cells were resuspended in 100 μl of DMEM and 1% BSA and placed in the upper compartment of the transwell unit. After 16 h of incubation at 37°C, cells were fixed in methanol and stained with Diff-Quick (Dade, Aguada, Puerto Rico). Nonmigratory cells in the upper surface of the filter were removed by wiping with a cotton swab. Phase-contrast microscopy at ×200 magnification was used to determine migration by counting cells that had migrated to the lower surface of the filter. Ten random fields were counted for each filter. Each sample was assayed in triplicate, and assays were repeated at least twice.

Cell invasion assays through Matrigel-coated filters were carried out by the method of Albin et al. (21). Transwell filters were coated with 20 μg of Matrigel per filter in 100 μl of cold DMEM to form a thin continuous layer on top of the filter. The filters were left to air dry overnight. The remainder of the assay was carried out as described above for the migration assay. To rule out possible cytotoxic effects of anti-HGF neutralizing antibody at the dosage applied in the migration and invasion assays, the viability of cells on both sides of the filters was determined by the trypan blue dye exclusion method.

Morphogenesis Assays. Three-dimensional Matrigel invasion assays were performed as described previously (22) with minor modifications. Samples of 10\textsuperscript{5} cells were suspended in 100 μl of DMEM in the presence or absence of 200 units/ml HGF/SF ± anti-HGF neutralizing antibody (10 μg/ml), placed on top of nonnululated growth factor-reduced Matrigel (14 mg/ml), and incubated at 37°C. Fresh medium was added after 36 h. After 3 days, the representative cell structures were photographed at ×400 magnification.

Northern Analysis. Total RNA was prepared from cell cultures according to the method described by Chomczynski and Sacchi (23). Fifteen μg of total RNA were subjected to electrophoresis in 1.2% formaldehyde agarose gels and transferred to Hybond-N+ nylon membrane (Amersham) by capillary effect using 10× SSC. After the transferred RNA was UV-cross-linked for 2 min, the membrane was prehybridized at 42°C for 24 h in a buffer consisting of 50% deionized formamide, 5× SSC, 1× Denhardt’s solution, 50 mM NaH\textsubscript{2}PO\textsubscript{4} (pH 6.5), 150 μg/ml single-stranded DNA, and 0.5% SDS. The membrane was then hybridized at 42°C overnight with 3 × 10\textsuperscript{6} cpm of the purified [\textsuperscript{32}P]cDNA probe in 12 ml of hybridization buffer (prehybridization buffer containing 20% dextran sulfate). After hybridization, the membrane was washed with 0.1% SDS and 0.1× SSC once for 5 min at room temperature and four times for 20 min at 60°C. Autoradiography was performed by exposing X-Omat-AR film (Kodak, Rochester, NY) to the hybridized membrane for 18 h at −70°C in the presence of an intensifying screen.

RESULTS

Expression of Met and HGF/SF in Human Gliomas. We examined sections of 32 human primary brain tumors by double immunofluorescence staining for Met and HGF/SF, using quantitative CLSM imaging (7, 13). All tumors were positive for Met staining, and 72%
showed HGF/SF staining (Table 1; Fig. 1, A–I). Met and HGF/SF costaining was observed in one-third of the low-grade astrocytomas, in 8 of 11 anaplastic astrocytomas (grade III), and in 13 of 15 glioblastoma multiformes (grade IV). Analysis of the staining intensity by the Optimas quantification procedure showed anaplastic astrocytomas to have the highest average Gy value for Met immunofluorescence (Table 1). By contrast, the average Gy value for HGF/SF staining was higher in the glioblastoma multiformes than in the anaplastic astrocytomas.

Met and HGF/SF staining was localized in cells resembling reactive astrocytes and in the large neurons in poorly defined normal brain tissue adjacent to the tumors (data not shown). Staining for Met and HGF/SF was also found in hypercellular and infiltrative areas of all anaplastic astrocytomas and glioblastoma multiformes examined (arrows in Fig. 1, C and I) and in characteristic pseudopalisading areas of glioblastoma multiformes (arrows in Fig. 1b). In addition to the infiltrating tumor cells, Met and HGF/SF staining was observed in the endothelial cells in perivascular and vascular areas of glioblastoma multiformes (arrows in Fig. 1f), whereas less intense Met and HGF/SF immunostaining was observed in areas of neovascularization in low-grade and anaplastic astrocytomas (data not shown). Whether the source of HGF/SF is autocrine or paracrine, these results suggest that high levels of Met-HGF/SF are expressed in the most aggressive type of brain tumors and that Met and HGF/SF expression correlates with the stage of the tumor.

Met and HGF/SF Expression in Glioma and NHA Cells in Vitro. We examined Met and HGF/SF expression in glioma cell lines and in NHA cells. By immunofluorescence staining, we showed that all of the glioma cell lines as well as the NHA cells expressed Met (Fig. 1, J and K). Met expression in these cell lines was confirmed by immunoprecipitation with the C-28 anti-Met antibody followed by Western analysis (Fig. 2a). These analyses show that all glioma cell lines as well as NHA cells express both p170 Met and p140 Met and that HGF/SF treatment increases the reactivity of p140 with anti-P-Tyr (Fig. 2a), similar to the Met-expressing SK-LMS-1 cells (20, 22). Moreover, the addition of exogenous HGF/SF resulted in the down-modulation of p140 Met in all of the glioma cell lines examined (Fig. 2b and data not shown). In the NHA cells, however, although anti-P-Tyr reactivity with Met increases in response to HGF/SF, no down-modulation of p140 was observed (Fig. 2c).

In most of the glioma cell lines, the background level of anti-P-Tyr reactivity after serum deprivation (Fig. 2a) suggested the possibility for HGF/SF autocrine signaling. Moreover, when we treated cells overnight with increasing amounts of anti-HGF/SF neutralizing antibody, the level of p140Met increases significantly in the glioma cells (Fig. 2d and data not shown), suggesting that receptor down-modulation might be occurring via an autocrine loop. However, the p140 of the NHA cells did not significantly increase under these conditions (Fig. 2d). We next examined both the glioma and NHA cells for hgf/sf RNA transcripts by Northern analysis and used RNA from the HGF/SF-expressing MRC-5 cells as a control (24). These analyses show the presence of hgf/sf-specific RNAs in the NHA and glioma cell lines (Fig. 3a). With the exception of the SW-1783 cell line, the two characteristic hgf/sf RNA transcripts of 6.3 and 3.1 kb (25) were readily detected in the U-118, U-138, U-373, and DBTRG cell lines, whereas lower levels were detected in the NHA cells.

By Western analysis, we next determined whether HGF/SF was present in the CM prepared from the cell lines. As expected in the MRC-5 cell supernatant, high-level expression of HGF/SF as p90 was observed under nonreducing conditions. However, HGF/SF was also detected in the CM of U-118 cells, and lower levels of expression were detected in fluids prepared from NHA and four other glioma cell lines (Fig. 3b). Moreover, metabolically labeled HGF/SF was detected in the cell supernatants at levels consistent with the levels detected by Western analysis (compare Fig. 3, b and c). Also consistent with the high levels of HGF/SF detected in the MRC-5 and U-118 glioma cell lines, we detected scatter factor activity in nonconcentrated CM of these two cell lines (data not shown). Collectively, these results indicated that the NHA and the glioma cells display autocrine Met-HGF/SF expression.

HGF/SF Is a Growth-stimulatory Factor for Glioma Cells but not for NHA Cells. We examined the effect of HGF/SF on thymidine incorporation by glioma and NHA cells after serum deprivation (Fig. 4a). For each of the glioma cell lines, we observed a decrease in [3H]thymidine uptake in the presence of anti-HGF/SF neutralizing antibody, suggesting that a low level of autocrine-stimulated incorporation occurred (Fig. 4a). With 50–200 units/ml HGF/SF, we observed dose-dependent increases in [3H]thymidine incorporation in all glioma cell lines, with a maximal effect at 100 or 200 units/ml. The level of [3H]thymidine incorporation ranged from slight in the DBTRG cells to high in the U-118 cell line. Interestingly, although significant increases in [3H]thymidine incorporation occurred in all glioma cell lines, the NHA cells were nonresponsive (Fig. 4a). Again, when these experiments were performed in the presence of 0.5, 2.5, and 5% serum, we observed HGF/SF-dependent [3H]thymidine incorporation in all of the glioma cell lines but not in the NHA cells (data not shown). Thus, although Met in NHA cells is tyrosine-phosphorylated in response to exogenous HGF/SF (Fig. 2a), Met is not down-modulated under these conditions (Fig. 2c), and the cells do not respond mitogenically to the ligand, nor is thymidine incorporation affected by anti-HGF neutralizing antibody (Fig. 4a). The control SK-LMS-1 cells responded to HGF/SF as expected (20).

HGF/SF Stimulates Glioma Cell Migration and Invasion in Vitro. HGF/SF is known to enhance the motility and invasiveness of a wide variety of cell types (5); therefore, we measured the influence of HGF/SF on migration (motility) of the glioma and NHA cells (Fig. 4b). As with the mitogenicity assay (Fig. 4a), the addition of anti-HGF neutralizing antibody to each of the glioma cell lines resulted in a reduced basal level of motility, whereas, in a dose-dependent manner, HGF/SF stimulated the motility of these cell lines up to 3.5-fold. Interestingly, a comparison of the mitogenic and motility responses of the glioma cells shows a negative correlation (Fig. 4, a and b), with DBTRG displaying the greatest migration, and U-118 cells displaying the least migration. Again, the NHA cells were nonresponsive (Fig. 4b).

We next performed invasion assays (21) with the NHA and the glioma cell lines (Fig. 4c). All of the glioma cells were highly invasive and, similar to the results of mitogenic and motility assays, anti-HGF neutralizing antibody significantly decreased their basal

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Table 1  Met-HGF/SF staining in primary human gliomas

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* The staining intensity was quantified by the Optimas quantification procedure obtained by confocal laser scan microscopy (19) from 32 cases of primary brain tumors that have been subjected to double immunofluorescence staining using antibodies to Met (C-28) and HGF/SF (7-11).

b Average Gy value for Met: 1*, 1–1.70; 2*, 71–140; and 3*, 141–210.

c Average Gy value for HGF/SF: 1*, 1–20; 2*, 21–50; and 3*, 51–80.

d Number of cases in the Gy value range/total examined.

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Fig. 1. Met and HGF/SF staining of human gliomas. Double immunofluorescence staining for HGF/SF and Met was analyzed by CLSM analysis (7, 20, 26). A–I, green, Met; red, HGF/SF; yellow, overlaid image of both Met and HGF. A–C, a section of anaplastic astrocytoma tissue with infiltrating tumor cells (arrows). D–F, an endothelial hyperplastic area of glioblastoma multiforme tissue with vascular and perivascular Met and HGF/SF staining in endothelial cells and tumor cells (arrows). G–I, Met and HGF/SF staining in a characteristic pseudopalisading area (arrows) of glioblastoma multiforme tissue. J and K, Met staining (green) with D1 (a mouse monoclonal antibody raised against human Met) in NHA and glioma (U-118) cells, respectively. The nuclei are counterstained with DAPI (red). Scale bars are included.
level of cell invasion; this antibody had no effect on the NHA cells (Fig. 4C). Thus, all of the glioma cells were mitogenic, motile, and invasive in response to exogenous added HGF/SF; however, the basal level of these responses was significantly reduced by anti-HGF antibody. Collectively, with the Met and HGF/SF expression studies (Figs. 1–3), these analyses strongly imply that an autocrine Met-HGF/SF stimulation occurs in all five glioma cell lines examined.

**Morphogenesis of Glioma Cells.** We subjected U-118 cells to morphogenesis assays in Matrigel. In the presence or absence of HGF/SF (Fig. 5, A and C), the morphological appearance of the U-118 cells did not change. However, when anti-HGF neutralizing antibody was added to the medium, the pattern changed significantly, and individual cells were larger and showed bipolar or tripolar morphology with long processes. By contrast, NHA cells did not exhibit any branching morphogenesis in the presence or absence of HGF/SF (data not shown). Moreover, in the absence of HGF/SF, these cells failed to grow and did not survive for more than 3 days, suggesting that the ligand is a survival factor for these cells in Matrigel.

**DISCUSSION**

We have demonstrated in mouse and human model systems that autocrine Met-HGF/SF activation is sufficient for tumorigenesis and metastasis in athymic nude mouse assays (13, 20, 22, 26). Intrinsic tumors of the central nervous system are characterized by their ability to infiltrate the adjacent brain parenchyma in a diffuse manner, often migrating several millimeters beyond any obvious tumor margin (1). Here we show that Met is expressed in all grades of human primary brain tumors, and two-thirds of these tumors are also immunoreactive for HGF/SF (Fig. 1; Table 1). We found that relative to Met, HGF/SF staining intensities were increased in the more aggressive tumors and, by quantitative image analysis, were higher in glioblastoma multi-
However, whereas HGF/SF was able to stimulate tyrosine phosphorylation of Met in NHA cells, neither growth, migration, nor invasion responses were obvious in these cells, and there was no effect of anti-HGF neutralizing antibody on basal activities of NHA cells in these assays (Fig. 4). The only phenotype was in Matrigel, in which HGF/SF permitted NHA cell survival. The mechanisms underlying the nonresponsive phenotype are unknown but could be very important for understanding abnormal Met-HGF/SF signaling in glioma cells.

Through biochemical and biological assays in vitro, we found that autocrine Met activation occurs in all of the glioma cell lines examined. For example, neutralizing anti-HGF antibody reduced the basal forms than in anaplastic astrocytomas. The correlation between HGF/SF immunoreactivity and grade of malignancy may be of prognostic value but may also suggest a role for HGF/SF-Met signaling in the malignant progression of glioblastoma multiformes from low-grade tumors. We observe distinct infiltrating tumor cells immunoreactive for both Met and HGF/SF invading the normal brain parenchyma (Fig. 1). We also show for the first time that HGF/SF and Met colocalize in tumor cells adjacent to or infiltrating the vessel walls (Fig. 1, D–F).

HGF/SF is a potent angiogenic factor in vivo and stimulates endothelial cell growth, motility, scattering, and branching morphogenesis in three-dimensional collagen gels (10, 27). Neovascularization is a hallmark of malignant gliomas, which is generally associated with hemorrhage, vein thrombosis, and tumor necrosis (1). Thus, the endothelial hyperplastic activity characteristic of glioblastoma multiformes is consistent with the potent angiogenic activity of HGF/SF in vivo (10). Whereas vascular endothelial growth factor is a potent endothelial-specific mitogen and also a potential angiogenic factor in human gliomas in vivo (28), we have shown that in vitro, HGF/SF does not influence vascular endothelial growth factor expression in our glioma cell lines (data not shown), suggesting that HGF/SF may independently stimulate angiogenesis. The colocalization in vivo of Met and HGF/SF in tumor vasculature suggests that autocrine and/or paracrine Met-HGF/SF signaling either in endothelial cells or adjacent tumor cells may play an important role in the neovascularization process in gliomas. Met and HGF/SF were also detected in NHA cells.
Urokinase-type plasminogen activator has been detected in glioma cells and tissues (17, 30). Tumor invasion is a complex multistep process that involves motility and degradation of matrix by proteolytic enzymes. Activation of the urokinase-type plasminogen activator proteolytic network by Met-HGF/SF signaling has been associated with the invasive metastatic phenotype of human tumor cells (22) and may be involved in glioma invasion. Curiously, U-118 cells, which express the highest level of HGF/SF, were most responsive mitogenically to HGF/SF but were least responsive in the migration and invasion responses compared with the other four glioma cell lines. The reasons for this variation are unknown, but if epigenetic variation of the levels of HGF/SF and Met expression influences the activities of proliferation and invasion, as suggested by Rong et al. (6), this could contribute to the poor prognosis of these tumors.

ACKNOWLEDGMENTS

We are grateful to Marianne Oskarsson, Richard Frederickson, and Ave Cline for technical assistance. We also thank Craig Webb, Michelle Fiscella, and Nicholas Duesbery for critical reading of the manuscript.

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