Expression and localization of scatter factor/hepatocyte growth factor in human astrocytomas[1]

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Scatter factor/hepatocyte growth factor (SF/HGF) is a pleiotropic cytokine that has been implicated in glioma invasion and angiogenesis. The SF/HGF receptor, MET, has been found to be expressed in neoplastic astrocytes as well as in endothelial cells of the tumor vasculature. Both SF/HGF and MET expression have also been described to correlate with the malignancy grade of human gliomas. However, most glioblastoma cell lines lack SF/HGF expression, raising the question of the cellular origin of SF/HGF in vivo. Using in situ hybridization, we analyzed glioblastomas, anaplastic astrocytomas, diffuse astrocytomas, pilocytic astrocytomas, and normal brain for the expression of SF/HGF mRNA. We detected strong SF/HGF expression by the majority of the tumor cells and by vascular endothelial cells in all glioblastoma specimens analyzed. Combined use of in situ hybridization with fluorescence immunohistochemistry confirmed the astrocytic origin of the SF/HGF-expressing cells. In contrast, CD68-immunoreactive microglia/macrophages, as well as vascular smooth muscle cells reactive to α-smooth muscle actin, lacked SF/HGF expression. In anaplastic, diffuse, and pilocytic astrocytomas, SF/HGF expression was confined to a subset of tumor cells, and signals were less intense than in glioblastomas. In addition, we detected SF/HGF mRNA in cortical neurons. SF/HGF expression was not up regulated around necroses or at tumor margins. MET immunoreactivity was observed in GFAP-expressing astrocytic tumor cells and endothelial cells as well as in a subset of microglia/macrophages. We conclude that in vivo, both autocrine and paracrine stimulation of tumor cells and endothelium through the SF/HGF-MET system are likely to contribute to tumor invasion and angiogenesis. Lack of SF/HGF expression by most cultured glioblastoma cells is not representative of the in vivo situation and most likely represents a culture artifact. *Neuro-Oncology 3, 82–88, 2001 (Posted to Neuro-Oncology [serial online], Doc. 00-044, February 19, 2001. URL <neuro-oncology.mc.duke.edu>)*

SF/HGF[3] stimulates the motility and proliferation of several cancer cell types and can induce angiogenesis (Rosen et al., 1994). Its biologic effects are mediated by the transmembrane tyrosine kinase receptor, MET, which is encoded by the proto-oncogene *MET*. Several studies suggest that, in gliomas, SF/HGF may function as an important tumor progression factor. We have previously observed a correlation between the concentration of SF/HGF in gliomas with the malignancy grade (Lamszus et al., 1998). Moreover, SF/HGF turned out to be a statistically independent predictive parameter for glioma microvessel density, independent of VEGF (Schmidt et al., 1999).

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[3]Abbreviations used are as follows: DIG, digoxigenin; GFAP, glial fibrillary acidic protein; SF/HGF, scatter factor/hepatocyte growth factor; α-SMA, α-smooth muscle actin; SSC, saline-sodium citrate; VEGF, vascular endothelial growth factor.

Glioblastoma cell lines usually express the MET receptor and are biologically responsive to SF/HGF. The establishment of an autocrine loop in rat and human glioblastoma cell lines resulted in the formation of significantly larger and more vascularized tumors in vivo (Laterra et al., 1997). Moreover, ribozyme-mediated knockdown of SF/HGF or MET expression in SF/HGF-secreting cell lines was shown to inhibit glioblastoma growth in vivo (Abounader et al., 1999). Although glioblastomas often contain high concentrations of SF/HGF in vivo, most glioblastoma cell lines do not express SF/HGF in vitro. This discrepancy raises the question of the cellular origin of SF/HGF in gliomas in vivo. Immunohistochemical studies have demonstrated SF/HGF and MET staining of tumor cells as well as staining of blood vessel endothelium in gliomas (Hirose et al., 1998; Koochekpour et al., 1997; Lamszus et al., 1999; Rosen et al., 1996). However, this observation does not necessarily imply that SF/HGF is also expressed by these cells because as a soluble protein, it could be bound to the cell surface after being secreted by other cell types, for example, microglial cells.

To analyze the expression and distribution of SF/HGF in human astrocytomas, we performed in situ hybridization on astrocytomas of all 4 malignancy grades (World Health Organization grades I to IV) (Kleihues and Cavenee, 2000) and on nontumorous brain tissue. To determine the exact cellular source of SF/HGF, we used a combined in situ hybridization/fluorescence immunohistochemistry double-labeling approach that allowed us to identify not only astrocytic cells but also microglia/macrophages and vascular smooth muscle cells. We further analyzed the cellular distribution of MET using double-labeling fluorescence immunohistochemistry.

Materials and Methods

Tissue Specimens

Tumor tissue was obtained from patients treated at the Department of Neurosurgery, University Hospital Hamburg-Eppendorf, Hamburg, Germany, and used for research with the informed consent of the patients. All tumors were from patients who had not been treated previously for their brain tumors. One normal brain specimen was obtained from a histologically noninfiltrated area of a lobectomy in a patient with a well-delineated right temporal tip low-grade astrocytoma (cases 8 and 13, Table 1), and another specimen of normal brain became available during decompression of an acute intracerebral hemorrhage. All tumors were classified according to current World Health Organization criteria (Kleihues and Cavenee, 2000). Specimens were fixed in 4% buffered formalin and embedded in paraffin.

Preparation of Probes

A 305-bp reverse transcriptase–polymerase chain reaction fragment corresponding to the first and second kringle domain of the SF/HGF cDNA (nucleotides 673 to 978) was subcloned into the *Xho*I and *Eco*RV sites of pBluescript II SK+/– (Stratagene, La Jolla, Calif.). The plasmid was linearized, and transcripts were synthesized from the T7 and T3 polymerase promoters using 1 μg plasmid as template, with DIG-labeled uridine triphosphate as substrate, according to the manufacturer's instructions (DIG RNA Labeling Kit; Roche, Mannheim, Germany). The yield was estimated by spotting serial dilutions of transcripts onto a nylon membrane, followed by detection with anti-DIG alkaline phosphatase-labeled antibody and visualization with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phospha te/levamisole (in 100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$) as described by the manufacturer. Labeled sense and antisense probes were stored at –80°C or used at once for in situ hybridization analysis.

In Situ Hybridization

Detection of SF/HGF mRNA was performed by following a protocol, with minor modifications, for nonradioactive

Table 1. Semiquantitative in situ hybridization and immunohistochemistry analysis

		SF/HGF in situ hybridization		MET immunohistochemistry	
Case No.	Tissue	Tumor cells	Endothelial cells	Tumor cells	Endothelial cells
1	Glioblastoma	+++	+++	+++	+++
2	Glioblastoma	++	+++	+++	++
3	Glioblastoma	+++	+++	++	+++
4	Glioblastoma	+++	++	+++	+++
5	Anaplastic astrocytoma	++	++	++	++
6	Anaplastic astrocytoma	++	++	++	+
7	Astrocytoma	+	+	+	+
8	Astrocytoma	+	+	++	+
9	Astrocytoma	+	+	+	++
10	Pilocytic astrocytoma	+	+	+	++
11	Pilocytic astrocytoma	++	+	+	+
12	Control brain		–		–
13	Control brain		–		–

+++, strong signal; ++ clear staining signal; + faintly recognizable over background; – no staining.

in situ hybridization (Komminoth, 1996). Sections were dewaxed, rehydrated, and after incubation with 0.3% Triton X-100 for 15 min, digested with Proteinase K (7.5 μg/ml) at 37°C for 30 min. Slides were postfixed in 4% paraformaldehyde and acetylated twice for 5 min each time with 0.1 M triethanolamine (pH 8) and 0.25% acetic anhydride. Sections were prehybridized for 30 min at 37°C using 4X SSC with 50% deionized formamide. Hybridization was carried out using 10 ng DIG-labeled antisense or sense probe in 30 μl hybridization buffer (4X SSC, 40% deionized formamide, 10% dextran sulfate, 1X Denhardt's solution, 10 mM 1,4-dithiothreitol, 1 mg/ml yeast t-RNA, and 1 mg/ml denatured and sheared herring sperm DNA). Slides were covered with plastic coverslips and incubated in a humidified chamber at 48°C overnight. Sections were washed twice for 15 min each time in 2X SSC and twice for 15 min each time in 1X SSC. To digest single-stranded unbound RNA probe, sections were incubated for 30 min at 37°C in NTE buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) containing 20 μg/ml RNase A. Subsequently, sections were again washed twice for 30 min each time in 0.1X SSC at 37°C. (Posthybridization washes at 50°C or 52°C in 2X SSC with 50% formamide were not found to be superior.) Sections were blocked with sheep serum in buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl containing 0.1% Triton X-100). Hybridized probes were detected by incubation with anti-DIG antibody conjugated to alkaline phosphatase diluted 1:500 at room temperature for 2 h. After washing, color was developed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate/levamisole solution for 4 to 12 h, after which color reactions were stopped, slides were rinsed in distilled water, and sections were mounted. Some sections were counterstained with nuclear Fast Red. Intensities of the in situ hybridization signals were scored on the basis of comparing different samples that were contained in 1 batch and were thus subjected to identical conditions. Two independent reviewers scored the intensities between "no staining," "faintly recognizable over background," "clear staining signal," and "strong signal."

Antibodies

Primary antibodies used were monoclonal antibodies against GFAP (IgG_1, dilution 1:30, M0761; DAKO, Hamburg, Germany), CD68 (IgG_1, dilution 1:100, M0876; DAKO), and α-SMA, (IgG_{2a}, dilution 1:400; Sigma, St. Louis, Mo.). Polyclonal rabbit anti-MET antibody (IgG, dilution 1:500) was obtained from Santa Cruz (C-28; Santa Cruz, Calif.). Secondary antibodies were fluorescein isothiocyanate–conjugated goat antimouse [$F(ab')_2$, dilution 1:30, F0479; DAKO], rhodamin-conjugated swine antirabbit (mainly IgG, dilution 1:30, R0156; DAKO), rhodamin-conjugated rabbit antimouse (mainly IgG, dilution 1:30, R0270; DAKO), and biotinylated goat antirabbit (5 μg/ml, BA-1000; Vector, Burlingame, Calif.).

Double-Labeling Fluorescence Immunohistochemistry

Four-micron-thick paraffin sections were dewaxed using standard histologic procedures. After microwave treatment, sections were blocked by using goat serum (Vector) and incubated simultaneously with polyclonal anti-MET and monoclonal anti-GFAP or anti-CD68 antibodies in buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% Triton X-100) at 4°C overnight. Sections were washed and incubated simultaneously with biotinylated goat antirabbit antibody and fluorescein isothiocyanate-conjugated goat antimouse antibody at room temperature for 1 h. After washing, slides were incubated with R-phycoerythrin–conjugated streptavidin (1:20, DAKO) at room temperature for 20 min. Thereafter, slides were washed and sections were mounted by using fluorescent mounting medium (DAKO). Sections were examined and photographed with a fluorescence microscope (Axiolab; Zeiss, Jena, Germany). Control sections were incubated with anti-MET, anti-GFAP or anti-CD68 alone, with the appropriate secondary antibodies as well as, alternatively, with the noncorresponding secondary antibodies to exclude cross-reactivity.

In Situ Hybridization/Fluorescence Immunohistochemistry Double-Labeling Analysis

To identify cells expressing SF/HGF, we first subjected slides to the in situ hybridization procedure as described above and subsequently to fluorescence immunohistochemistry using primary antibodies against GFAP, CD68, and α-SMA. Primary antibodies were detected using rhodamin-conjugated secondary antibodies against mouse or rabbit IgG as described above for the double-labeling immunohistochemistry. Sections were mounted using fluorescent mounting medium and visualized using a fluorescence microscope.

MET Immunohistochemistry

Immunostaining for MET with 3′3-diaminobenzidine as substrate was performed as described previously (Lamszus et al., 1999), and staining intensities were scored by 2 independent reviewers.

Results

Four neurosurgically resected glioblastomas, 2 anaplastic astrocytomas, 3 diffuse astrocytomas, 2 pilocytic astrocytomas, and 2 specimens of normal brain were analyzed by in situ hybridization for SF/HGF expression. Glioblastomas were selected for the presence of both necroses with pseudopalisading of tumor cells and microvascular proliferations. One of the glioblastoma cases had been included in a previous study (Schmidt et al., 1999) in which it had been found to contain very high levels of SF/HGF (4.2 ng/mg protein, measured by enzyme-linked immunosorbent assay) relative to other tumors.

In all 4 glioblastoma specimens, strong expression of SF/HGF was observed in most of the tumor cells (Fig. 1A and 1B; Table 1). In addition, specific SF/HGF hybridization signals were detected in endothelial cells of small, medium sized, and large tumor vessels (Fig. 1C, 1D, and 1E; Table 1). Hybridization signals were also detected in the endothelium of glomeruloid microvascular prolifera-

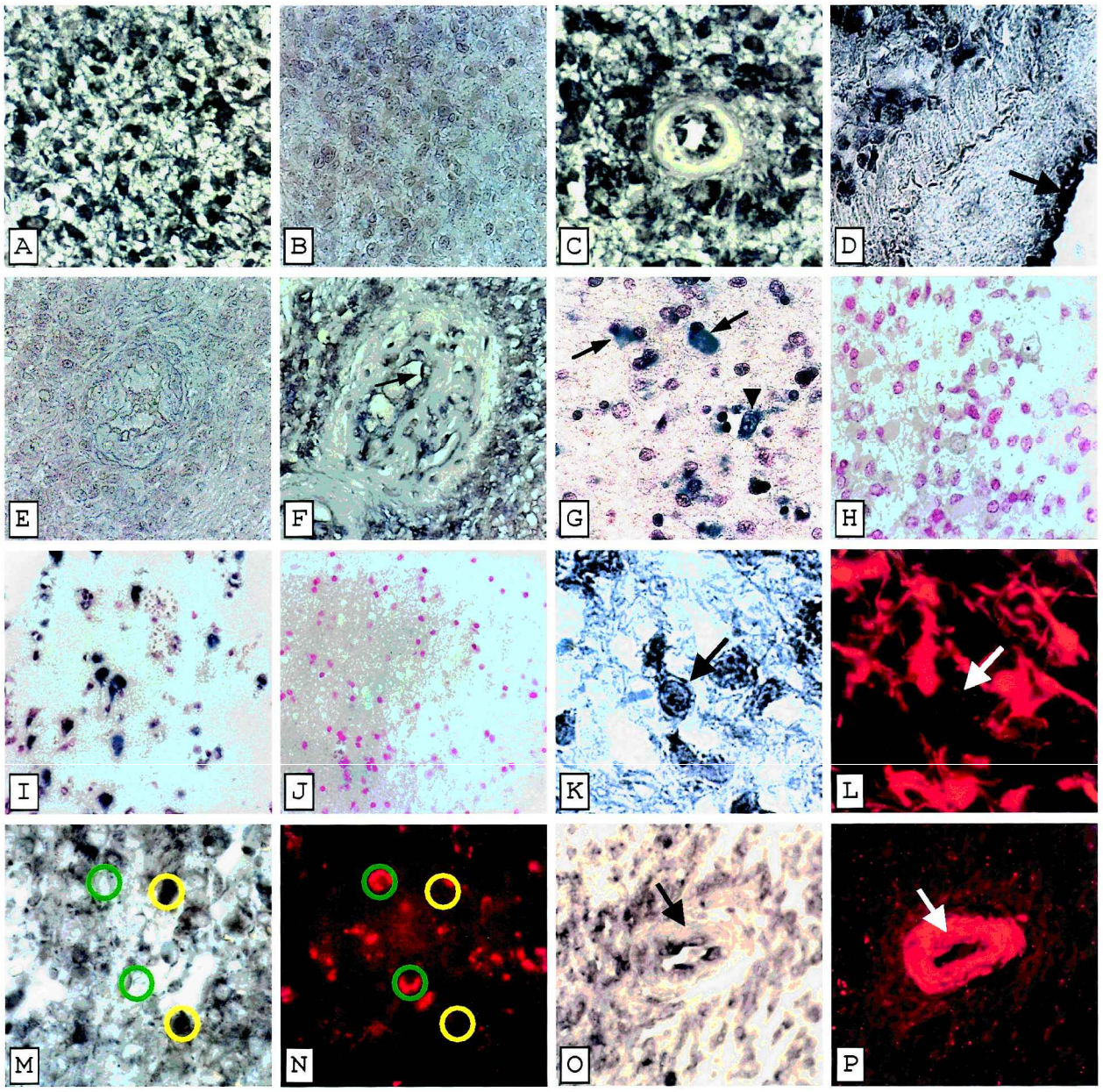

Fig. 1. Localization of SF/HGF expression in astrocytomas and normal brain. A. In situ hybridization of a glioblastoma showing SF/HGF mRNA expression by most tumor cells (original magnification ×200). B. Sense-control hybridization of the same glioblastoma (original magnification ×200). C. SF/HGF expression by endothelial cells of a small tumor vessel (original magnification ×300). D. SF/HGF expression by the endothelium (arrow) of a large intratumoral artery, and lack of SF/HGF expression by other cells of the vessel wall (original magnification ×280). E. Sense control hybridization of a tumor blood vessel demonstrating specificity of the endothelial staining (original magnification ×225). F. Hybridization signal for SF/HGF in the endothelium of a glomeruloid microvascular proliferation (arrow) (original magnification ×220). G. Anaplastic astrocytoma showing hybridization signals in diffusely infiltrating tumor cells (arrows) and also in a cortical neuron (arrowhead) (original magnification ×225). H. Sense control of the same anaplastic astrocytoma (original magnification ×225). I. SF/HGF expression in neurons of normal brain (original magnification ×170). J. Lack of SF/HGF expression in white matter astrocytes and oligodendrocytes of normal brain (original magnification ×170). K and L. Hybridization signal for SF/HGF in an individual astrocytic tumor cell (K, arrow; and confirmation of its astrocytic origin by subsequent GFAP immunofluorescence staining (L, arrow) (both original magnification ×700). M and N. Green circles surround microglial/macrophagic cells identified by CD68 immunofluorescence (N), which are intermixed with the tumor cells in a glioblastoma, and which lack SF/HGF mRNA expression (M). Conversely, cells marked by yellow circles, which most likely represent tumor cells, show SF/HGF expression (M) but are CD68 negative (N) (both original magnification ×300). O and P. Immunofluorescence staining for α-SMA identifying vascular smooth muscle cells (P, arrow), and confirming that, in contrast to endothelium, these cells do not express SF/HGF (O, arrow) (both, original magnification ×210). G, H, I, and J were counterstained with nuclear Fast Red. Photos A-F and K-P were taken from case 1; G-H were taken from case 5; and I-J were taken from case 12 (also see Table 1).

tions (Fig. 1F). SF/HGF expression was relatively homogenously distributed throughout the tumor sections and was not particularly up regulated around necrotic areas or at the tumor margin.

In anaplastic astrocytomas, diffuse astrocytomas, and pilocytic astrocytomas, SF/HGF expression was confined to a subset of tumor cells with clear hybridization signals that were not as strong as those in glioblastomas (Fig. 1G and H; Table 1). Likewise, the hybridization signals in the endothelial cells of vessels in low-grade gliomas were weaker in comparison to those in highly malignant gliomas (Table 1). Interestingly, in tumor-infiltrated cortex, we observed neuronal SF/HGF expression (Fig. 1G and 1H). SF/HGF expression by neurons was also detected in sections of nontumorous brain (Fig. 1I), whereas SF/HGF mRNA was undetectable in normal astrocytes, oligodendrocytes, or endothelium of nontumorous brain (Fig. 1J; Table 1).

Whereas structures such as blood vessel endothelium can easily be identified morphologically, glial tumor cells can be difficult to identify clearly, because these cells are often intermixed with microglia/macrophages (Roggendorf et al., 1996). In particular, microglial cells can be morphologically indistinguishable from tumor cells by conventional histologic analysis. We therefore applied an in situ hybridization/fluorescence immunohistochemistry double-labeling analysis to the tumor sections to determine the exact cellular source of SF/HGF. In situ hybridization for SF/HGF mRNA, combined with immunofluorescence staining for GFAP, confirmed the expression of SF/HGF by most astrocytic tumor cells in glioblastomas (Fig. 1K and 1L). In contrast, when we used an antibody against CD68, microglia/macrophages that were intermixed with the tumor cells were SF/HGF-negative (Fig. 1M and 1N). Moreover, by using the same double-labeling technique with an antibody against α-SMA, we also found that the vascular smooth muscle cells did not express SF/HGF (Figs. 1O and 1P).

To identify cells expressing the transmembrane receptor MET, we used double-labeling immunofluorescence. Double staining with anti-CD68 showed that a subset of the microglia/macrophages, present in between the tumor cells, were immunoreactive for MET (Figs. 2A and 2B). Combined staining for GFAP and MET showed that most tumor cells in glioblastomas and low-grade gliomas expressed the MET receptor (Fig. 2C and 2D). In addition, MET immunoreactivity was observed on endothelial cells of intratumoral blood vessels (not shown). In the more malignant gliomas, staining was more uniformly distributed and more intense compared with low-grade tumors.

Discussion

The results of this study indicate that, in astrocytic tumors, SF/HGF expression is confined to tumor cells and endothelial cells. In addition, increasing SF/HGF expression is observed with increasing tumor grade. In combination with the presence of immunohistochemically detectable MET protein on tumor cells and endothelial cells, this finding suggests that both autocrine and paracrine stimulation of tumor cells and endothelium are relevant to tumor progression and angiogenesis. In contrast, a subset of microglia/macrophages may be influenced in a paracrine fashion, because CD68-positive cells lack SF/HGF expression but are sometimes immunoreactive for the MET receptor.

Using enzyme-linked immunosorbent assay, we previously observed an increase in total SF/HGF concentration in malignant gliomas compared with low-grade tumors (Lamszus et al., 1998). Similarly, the intensity of MET immunostaining in tumor cells is elevated in high-grade gliomas compared with low grade gliomas (Hirose et al., 1998; Koochekpour et al., 1997; Rosen et al., 1996). However, immunohistochemistry does not allow the identification of the exact SF/HGF source. Our results now show that SF/HGF ligand is produced by neoplastic astrocytes. These findings are consistent with the loss of SF/HGF expression by most cultured glioblastoma cell lines as being an in vitro artifact.

Detailed studies of SF/HGF and *MET* mRNA expression patterns have previously been performed on normal brain of different mammalian species, but not on gliomas. Using in situ hybridization, both SF/HGF and *MET* expression were detected mainly in neurons of developing and adult rat brain as well as in human brain (Achim et al., 1997; Jung et al., 1994). Neuronal SF/HGF was demonstrated immunohistochemically as well

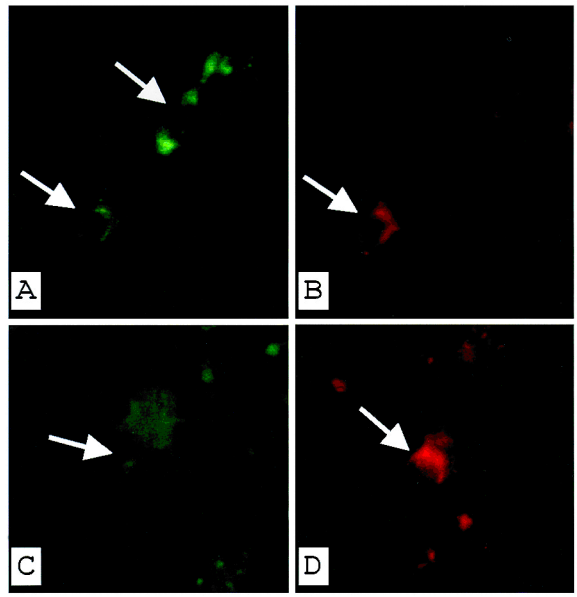

Fig. 2. Double fluorescence immunohistochemistry for MET and for CD68 or GFAP. A. Microglial/macrophagic cells identified by immunoreactivity for CD68 (arrows). B. One CD68 positive cell displaying surface immunofluorescence for MET (arrow), with the other being negative (both, original magnification ×825). C. An astrocytic tumor cell identified by GFAP immunofluorescence (arrow). D. The same cell displaying MET immunoreactivity (arrow) (both, original magnification ×560). Note that in the vicinity of the astrocytic cell some cells or cellular processes can be identified that lack GFAP expression and most likely correspond to microglia/macrophages. These photos were taken from case 4 (also see Table 1).

(Zarnegar et al., 1990). In addition, coexpression of SF/HGF and *MET* mRNA have also been detected in sympathetic neurons of the murine superior cervical ganglion (Yang et al., 1998), which together with several functional studies (Ebens et al., 1996; Maina et al., 1997; Wong et al., 1997; Yamamoto et al., 1997) suggests that SF/HGF acts as a survival and growth factor for neurons of various locations. We have confirmed the neuronal expression of SF/HGF in both normal tissue and in tumor-infiltrated cortical areas in the present study.

Whereas in situ hybridization studies on mammalian brain did not detect *MET* mRNA expression in microglial cells (Jung et al., 1994), immunohistochemical analysis showed MET protein on neurons as well as microglial cells (Di Renzo et al., 1993; Yamada et al., 1994). Such discrepancies could be due to variations in mRNA stability or differences in posttranscriptional regulation. Based on the microglial MET immunoreactivity, a paracrine relationship between neurons as source of SF/HGF and microglia as target cells has been proposed (Di Renzo et al., 1993). This view was supported by a recent functional analysis in which SF/HGF secreted by glioblastoma cells was able to chemotactically attract isolated microglial cells in vitro (Badie et al., 1999). Microglial cells are known to secrete numerous growth factors and cytokines, including interleukin-1, interleukin-6, and interleukin-10, basic fibroblast growth factor, tumor necrosis factor-α, and transforming growth factor-β (Moore and Thanos, 1996), and may therefore promote tumor growth. Our finding of MET immunoreactivity of a subset of intratumoral microglia/macrophages suggests that a mutual paracrine relationship may exist between neoplastic astrocytes and intratumoral microglia/macrophages.

SF/HGF expression by endothelial cells further substantiates the previously suggested involvement of this system in glioma angiogenesis. Similar to other angiogenic growth factors, such as VEGF or basic fibroblast growth factor, SF/HGF is able to induce angiogenesis in vivo and exert stimulating effects on endothelial migration and proliferation in vitro (Grant et al., 1993). In contrast to VEGF, which is secreted by the tumor cells and acts specifically on vascular endothelial cells, the SF/HGF-MET system appears to be activated in an autocrine fashion in endothelial cells. Also in contrast to VEGF, which is preferentially expressed around necrotic areas in glioblastomas (Plate et al., 1992; Phillips et al., 1993), we found SF/HGF mRNA to be distributed more evenly throughout the tumor without preferential up regulation around necroses. VEGF is a hypoxia-inducible factor (Shweiki et al., 1992), whereas it was recently reported that hypoxia can down regulate SF/HGF expression in endothelial cells and vascular smooth muscle cells (Hayashi et al., 1999). These observations suggest that different angiogenic growth factors are differentially regulated in gliomas and that angiogenesis can be induced by independent mechanisms. Moreover, the present results are in good agreement with our previous observation that both SF/HGF and VEGF have a predictive value for glioma angiogenesis that is statistically independent of each other (Schmidt et al., 1999). From a therapeutic standpoint, this may have complex implications, as interference with only one angiogenic mechanism might be of insufficient efficacy.

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