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Up-regulation of Vascular Endothelial Growth Factor Induced by Hepatocyte Growth Factor/Scatter Factor Stimulation in Human Glioma Cells

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Recent findings suggest that hepatocyte growth factor/scatter factor (HGF/SF) contributes to the malignant progression of human gliomas. We investigated the effect of HGF/SF on vascular endothelial growth factor (VEGF) expression of c-Met/HGF receptor-positive human glioma cell lines. Treatment of the glioma cells with various concentrations of HGF/SF resulted in an enhanced secretion of VEGF proteins accompanying increased transcription of VEGF mRNA in a dose-dependent fashion. Since malignant gliomas frequently co-express HGF/SF and its receptor, these results suggest that HGF/SF could act as an indirect angiogenic factor through autocrine induction of VEGF expression and secretion in malignant gliomas. © 1998 Academic Press

Vascular endothelial growth factor (VEGF) is a powerful specific mitogen for vascular endothelial cells [1, 2], originally purified on the basis of its vascular permeability-enhancing activity *in vivo*, hence its alternative name, vascular permeability factor (VPF) [3, 4]. VEGF/VPF is a glycosylated dimeric protein of approximately 46 kDa with structural homology to platelet-derived growth factor (PDGF). It exerts its function through binding to its receptors, VEGF-R1 (originally named as FLT) and VEGF-R2 (also known as flk-1, NYK and KDR), on the surface of the endothelial cells [5-7].

Gliomas are the most common type of human brain tumor. Glioblastoma is a high-grade glioma of astrocytic origin and is characterized by high vascularity, peritumoral edema, extensive invasiveness and rapid growth. The prognosis for patients with glioblastoma is poor, and at present, no successful treatment is available. The molecular events which underlie glioma progression have not been clarified, but abnormalities of p53 gene and amplification and overexpression of the epidermal growth factor (EGF) receptor gene may be involved [8]. Studies performed during the past several years revealed that VEGF/VPF plays an important role in the angiogenic property and growth of glioblastomas *in vivo*. For instance, VEGF/VPF mRNA is highly expressed in glioblastoma cells, particularly in the cells along necrotic areas [9-12], and VEGF/VPF protein is present in human glioma cell lines and in the surgical specimens of glioblastoma multiforme [11]. The levels of VEGF/VPF mRNA correlated significantly with vascularity in gliomas [13]. Furthermore, mRNAs for VEGF-R1 and -R2 are co-expressed in vascular cells in glioblastoma multiforme but not in normal adult brain [11, 14], and ectopic expression of antisense VEGF/VPF resulted in an inhibition of growth of glioma cells *in vivo* [15].

Hepatocyte growth factor (HGF) is a pleiotropic factor initially identified as a polypeptide growth factor for hepatocyte [16, 17], and is indistinguishable from scatter factor (SF) [18-20]. The receptor for HGF/SF is c-Met receptor tyrosine kinase, encoded by the *c-met* proto-oncogene [21, 22]. Evidences are accumulating in favor of the notion that HGF/SF and its receptor play an important role in progression of cancers of the systemic organs [23]. Previously, we reported that human glioblastoma cells co-express HGF/SF, its receptor and HGF activator [24]. Subsequent findings by a number of groups have suggested that HGF/SF contributes to the malignant progression of human gliomas [24-31] and may have a role in angiogenesis of gliomas *in vivo* [30]. Hypervascularity is one of the most important feature of human glioblastomas. Since VEGF/VPF appears to be the principle angiogenesis factor in gliomas,

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we examined the effects of HGF/SF on VEGF/VPF expression of c-Met positive human glioma cells. We now demonstrate that HGF/SF significantly stimulates VEGF/VPF expression in the glioma cells.

MATERIALS AND METHODS

Cell culture. Human glioma cell lines, U251 and KG-1-C were obtained from RIKEN cell bank (Tsukuba, Japan). Two human glioblastoma cell lines, MGM-1 and MGM-3 were established in our laboratory [24, 31]. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 20 mM HEPES buffer at 37°C in a humidified atmosphere containing 5% CO_2.

Cell treatment. Recombinant human HGF/SF (rHGF) and recombinant human EGF (rEGF) were obtained from Mitsubishi Chem. (Yokohama, Japan) and Becton Dickinson (Bedford, MA), respectively. Confluent cultures of each glioma cell line in quadruplicate 35-mm dishes were washed three times with serum-free medium and further incubated with 2 ml of DMEM containing 2% FBS or serum-free DMEM containing 0.1% bovine serum albumin (BSA), in the presence or absence of varying concentration of rHGF or rEGF. After incubation for 24 h, the conditioned media were harvested and centrifuged at 1500 × *g* for 20 min to remove cellular debris, and total RNA was extracted from the cells in two dishes as described below. The remaining two dishes were used to count the number of cells cultured. The supernatant was stored at -40°C until subsequent enzyme immunoassay.

To mimic hypoxic state, U251 cells were cultured in the presence of 10 or 100 μM of $CoCl_2$ (Sigma, St. Louis, MO) for 24 h or 48 h in serum-free DMEM containing 0.1% BSA in a humidified 5% CO_2/95% air atmosphere at 37°C. The levels of induced VEGF protein in the conditioned media were compared to those of the cells incubated in the absence of $CoCl_2$ with or without 20 ng/ml rHGF.

Enzyme immunoassay. Amounts of VEGF/VPF protein in the cultured conditioned media were measured by sandwich enzyme-liked immunosorbent assay (EIA) with a human VEGF EIA kit (Immuno-Biological Laboratories Co. Ltd., Gunma, Japan) according to the manufacturer's instruction. Immunoreactive HGF/SF proteins in the culture conditioned media were also measured by EIA assay kit (Otsuka Seiyaku, Tokyo, Japan).

Northern blotting. Total RNA was extracted from the cultured cells by using Trizol (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instruction, then 30-μg each of total RNA were electrophoresed on 1% formaldehyde agarose gel and transblotted onto Hybond-N^+ nylon membrane (Amersham, Buckinghamshire, U.K.) and RNA was UV-crosslinked onto the membrane. Hybridization was performed in mixed solution of 50% formamide, 5X Denhardt's solution, 25 mM phosphate buffer (pH 6.5), 0.1% SDS, 100 μg/ml of sonicated and heat-denatured salmon sperm DNA, and 5X standard saline-citrate (SSC) at 42°C for 16 h. The blots were washed as follows: 3 times in 0.1% SDS in 1X SSC for 15 min at room temperature; and twice in the same solution for 20 min at 65°C. The membranes were autoradiographed with Kodak XR-5 film at -80°C for 6 h or 18 h. The VEGF/VPF cDNA corresponding to bases -16 to 742, in which bases were numbered from the initiating ATG of the coding sequence for $VEGF_{165}$, was synthesized by reverse-transcriptase/polymerase-chain reaction (RT-PCR), subcloned into pCR II vector (Invitrogen, San Diego, CA), sequenced and used as a probe. For internal control of loading, the blots were subsequently hybridized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Clontech, Palo Alto, CA). The probes were radiolabeled by random priming with ^{32}P-CTP. For quantification of the northern blot, the radioactivities of the mRNA signals for VEGF/VPF were measured directly by a Bioimaging Analyzer, FUJIX BAS2000 system (Fuji Photo Film, Tokyo, Japan), and normalized by division through those of the corresponding GAPDH mRNA signals.

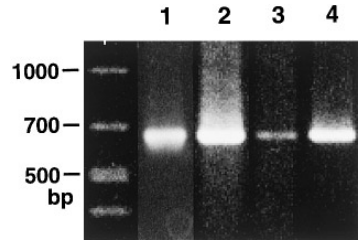

FIG. 1. RT-PCR for *c-met* mRNA. Total RNAs derived from cultured human glioma cell lines were used. A single specific band of 656 bp product was observed in each lane. Lane 1, U251; lane 2, KG-1-C; lane 3, MGM-1; lane 4, MGM-3.

RT-PCR for c-met. Expression of *c-met* mRNA by glioma cell lines was assayed by RT-PCR analysis. The forward and reverse primers used are 5′-ACAGTGGCATGTCAACATCGCT-3′ and 5′-GCTCGGTAGTCTACAGATTC-3′, respectively, and the predicted product size was 656 bp. For cDNA synthesis, 1 μg of total RNA was reverse-transcribed with random primer. For PCR, the cDNA samples were brought to a final concentration of 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs and 7 mM anti-Taq polymerase monoclonal antibody (Clontech). In addition, each sample contained 0.1 pmol of both reverse and forward primers and 2.5 U of Taq polymerase (Takara Shuzo, Shiga, Japan). PCR was carried out in a programmable heating block (Perkin Elmer Cetus, Norwalk, CT) using 5 cycles consisting of denaturation at 94°C for 1 min, annealing at 58°C for 2 min and extension at 72°C for 2 min, followed by 25 cycles consisting of 94°C for 30 sec, 58°C for 1 min and 72°C for 2 min. Products were analyzed by 2.5% agarose gel electrophoresis.

RESULTS

Expression of c-met gene in glioma cells and secretion of VEGF/VPF protein. Prior to the experiments, expression of *c-met* gene in the glioma cell lines was confirmed by means of RT-PCR analysis. As shown in Fig. 1, *c-met* mRNA was detectable in all cell lines used. In addition, among the cell lines, KG-1-C secreted a notable amount of HGF/SF protein. All cell lines secreted VEGF/VPF into the serum-free medium and the amounts of the secreted proteins are summarized in Table 1.

Effects of HGF/SF on the secretion of VEGF/VPF in glioma cell lines. Stimulatory effects of HGF/SF on the secretion of VEGF/VPF protein by the glioma cells were analyzed. Upon stimulation, secretion of VEGF/VPF by U251 cells increased in a dose-dependent manner (Fig. 2A). EGF is a well-known VEGF/VPF inducer for glioma cells, and according to the previous report, maximal stimulation was observed at 10 ng/ml (∼1.7 nM) [32]. The effect of HGF/SF on VEGF/VPF induction was comparable to or even superior to rEGF (Fig. 2A). U251 demonstrated 1.5, 1.8. 3.6 and 4.3-fold increase in the secretion of VEGF protein at concentrations of 0.12, 0.24, 1.2 and 2.4 nM of rHGF, respectively, and in this range the extents of the stimulation

TABLE 1

Secretion of VEGF/VPF and HGF/SF by Human Glioma Cells

Cell	VEGF/VPF (pg/ml)	VEGF/VPF (pg/10^6/24 h)	HGF/SF (pg/10^6/24 h)
U251	1600	1836	<
MGM-1	480	237	246
MGM-3	3840	5760	<
KG-1-C	234	1816	14600

Note. Confluent cells were cultured in serum-free DMEM containing 0.1% BSA (for VEGF/VPF) or DMEM containing 10% FBS (for HGF/SF), for 24 h and conditioned media were harvested and analyzed.

were statistically significant. Cobalt treatment, that mimics the hypoxia of the cells, also stimulate VEGF/VPF secretion significantly in U251 cells, and the effect of 0.24 nM rHGF was comparable to that of 10 μM $CoCl_2$ treatment (Fig. 2B). The treatment of MGM-1, MGM-3 and KG-1-C with rHGF (0.24 nM which corresponds to ~20 ng/ml) also resulted in an induction of VEGF/VPF (Fig. 2C). The extent of the stimulation was statistically significant in MGM-1 and MGM-3, but not in KG-1-C. Since only KG-1-C secreted notable amounts of HGF/SF into the culture supernatant (Table 1), it can be hypothesized that an autocrine stimulation may mask the effects of an exogenously added HGF/SF in KG-1-C cells.

Effects of HGF/SF stimulation on expression of VEGF/VPF mRNA in glioma cells. After treatment of U251 cells with various concentrations of HGF/SF (0.012-0.6 nM), the levels of specific mRNA for VEGF/VPF were investigated by means of Northern blot analysis. A main transcript around 4.0 kb was detected in U251 cells. VEGF/VPF mRNA level was increased in response to rHGF stimulation in a dose-dependent manner (Fig. 3). U251 cells showed 1.4, and 2.1-fold increase in VEGF/VPF mRNA levels at concentrations of 0.12 and 0.6 nM of rHGF, respectively, in the serum-free condition. In the presence of 2% FBS, U251 demonstrated 1.8 and 2.2-fold increase in VEGF/VPF mRNA levels at concentrations of 0.12 and 0.6 nM of rHGF, respectively.

DISCUSSION

HGF/SF is a pleiotropic factor and functions as a mitogen for variety of cell types, as a morphogen and as a motogen for some epithelial cells [23]. It is thought to be mainly a paracrine factor produced by cells of mesenchymal origin and acting on epithelial cells, including carcinoma cells, which bear its receptor [23]. Previously, we showed that human gliomas concomitantly express HGF/SF, *c-met* genes and HGF-activator, a serine proteinase that activates single chain HGF/SF precursor [24]. Subsequently, a number of groups, including ourselves, have reported that HGF/SF acts as a motility factor in glioma cells, stimulates matrix metalloproteinase production and also stimulates *in vitro* growth of the glioma cells [25-31]. *In vivo*, glioblastomas contain higher levels of HGF/SF protein than do either lower grade gliomas or normal brain [31]. Immunohistochemical analyses revealed that expression of c-Met and/or HGF/SF proteins is correlated with grade of malignancy in human astrocytic tumors [26, 28, 29, 34]. Genes for the ligand (HGF/SF) and receptor (c-Met) were preferably co-expressed in glioblastomas [24, 35]. These lines of evidence suggest that HGF/SF could have an important role in the malignant

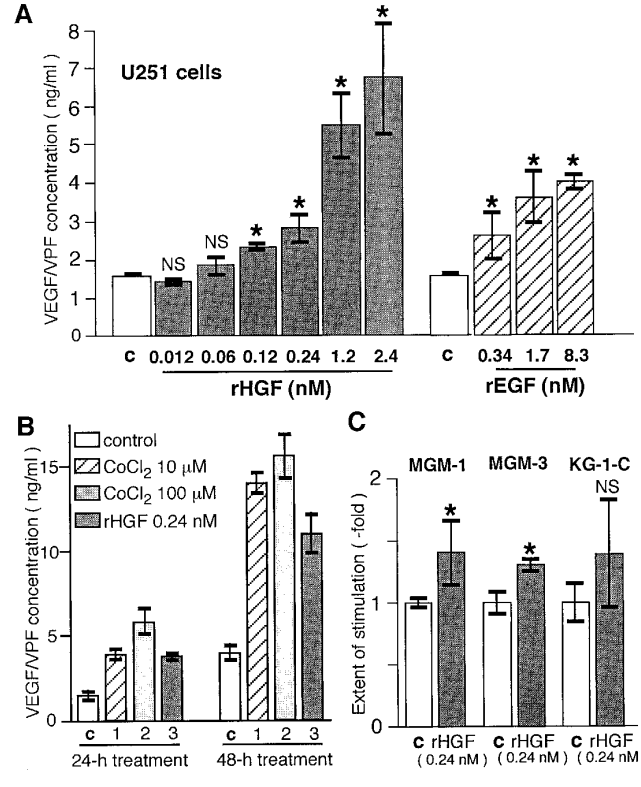

FIG. 2. Effects of HGF/SF on VEGF/VPF protein secretion in glioma cells. (A) U251 cells were treated with 0.012-2.4 nM (1-200 ng/ml) rHGF or 0.34-8.3 nM (2-50 ng/ml) rEGF. After 24 h, the conditioned medium was harvested and the concentration of immunoreactive VEGF/VPF protein was measured by sandwich EIA and compared to that of non-treated control U251 cells (c). (B) Effects of $CoCl_2$ treatment (24- or 48-h treatment) on VEGF/VPF secretion in U251 cells. Column c, non-treated control; column 1, 10 μM $CoCl_2$ treatment; column 2, 100 μM $CoCl_2$ treatment; column 3, 0.24 nM rHGF treatment. (C) Effects of rHGF on VEGF/VPF secretion of MGM-1, MGM-3 and KG-1-C glioma cell lines. Values were expressed as mean concentration of triplicated assay ± SD (in A and B), or as relative concentration (mean of triplicated assay ± SD) with that of the non-treated control arbitrarily set as 1.0 (in C). *:$p < 0.05$ compared to the control.

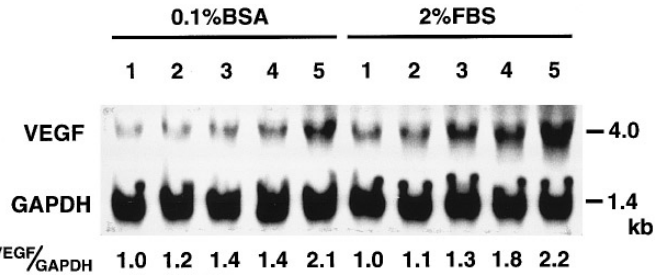

FIG. 3. Effects of HGF/SF on VEGF/VPF gene expression in glioma cells. Cultured U251 cells were incubated without (control, lane 1), or with 0.012 nM (lane 2), 0.06 nM (lane 3), 0.12 nM (lane 4) and 0.6 nM (lane 5) of rHGF for 24 h in serum-free DMEM containing 0.1% BSA (0.1% BSA) or in DMEM containing 2% FBS (2% FBS). The signals of VEGF/VPF mRNA were normalized by the corresponding GAPDH mRNA signals (VEGF/GAPDH), and the values are depicted in the figure (bottom), with that of non-treated U251 cells (lane 1) arbitrarily set at 1.0.

progression of human gliomas, and the autocrine loop of HGF/SF signaling pathway can be created in malignant gliomas, particularly in glioblastomas. Furthermore, since neural microvascular endothelial cells (NMVEC) also produced HGF/SF *in vitro* [26], and conditioned media from both glioblastoma and NMVEC contained HGF/SF inducing activity [26], augmented paracrine circuit of HGF/SF signaling pathway could be generated in a glioblastoma tissue as the result of mutual interaction between glioblastoma cells and endothelial cells.

In this report, we demonstrated that HGF/SF significantly induced VEGF/VPF expression and secretion by glioma cells bearing c-Met/HGF receptor. Of importance in this study, the effect of HGF/SF on the induction of VEGF/VPF was comparable to or even superior to EGF, a potent VEGF/VPF inducer in glioma cells [33], and was also comparable to cobalt treatment which can mimic hypoxic state of the cells. The induction and maintenance of tumor blood vessels are essential requirement for the growth of solid tumors including glial tumors, and human glioblastomas are known to be one of the most highly vascularised tumors. In gliomas, particularly glioblastomas, it appears that VEGF/VPF is the principle angiogenic factor derived from tumor cells and acting on endothelial cells, and that tumor growth-induced hypoxia plays a significant role in the induction of VEGF/VPF expression [9-15]. Recently, Laterra et al. described that overexpression of HGF/SF in human glioblastoma cells resulted in an enhanced tumorigenicity and growth *in vivo*, though the expression did not alter *in vitro* proliferation of the cells [36]. Moreover, HGF/SF gene transfer to rat 9L gliosarcoma cells enhanced tumor-associated angiogenesis [30] and HGF/SF stimulates endothelial cell proliferation and migration [26, 31, 37]. Therefore it seems likely that HGF/SF contributes to the angiogenesis and growth of glioblastomas *in vivo*. In this report, we obtained additional evidence for the contribution of HGF/SF to the angiogenesis in human gliomas, i.e., HGF/SF can stimulates the angiogenesis of glioma via autocrine up-regulation of VEGF/VPF expression in the glioma cells. A number of growth factors and cytokines as EGF, fibroblast growth factor (FGF), PDGF, tumor necrosis factor α, transforming growth factor β, or interleukin 1β have been reported to induce the expression of VEGF/VPF in a variety of cultured cells including glioma cells [33, 38-40]. Creation of FGF-4 autocrine loop in mouse mammary cells resulted in autocrine up-regulation of VEGF/VPF expression [41]. To date, effects of HGF/SF on VEGF/VPF expression of glioma cells remains to be clarified. In contrast to our present observation, it was described that HGF/SF did not influence VEGF/VPF expression in glioma cells *in vitro*, though the data and experimental procedure were not shown [28]. Very recently, it was reported that HGF can up-regulate VEGF/VPF expression of vascular smooth muscle cells [42].

In summary, our observation showing that HGF/SF up-regulates VEGF/VPF in glioma cells in well-oxygenated culture condition suggests that, in addition to a physiological process initiated by neoplastic overgrowth-induced hypoxia [9, 10], the genetic alteration such as a creation of autocrine (or paracrine) loop of HGF/SF signaling pathway in glioma cells also induces autocrine up-regulation of VEGF/VPF. This finding supports the hypothesis that expression of HGF/SF and/or its receptor, c-Met, in glioma cells contributes to the malignant progression of human gliomas.

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