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Glioma Inhibition by HGF/NK2, an Antagonist of Scatter Factor/Hepatocyte Growth Factor

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Strategies that antagonize growth factor signaling are attractive candidates for the biological therapy of brain tumors. HGF/NK2 is a secreted truncated splicing variant and potential antagonist of scatter factor/hepatocyte growth factor (SF/HGF), a multifunctional cytokine involved in the malignant progression of solid tumors including glioblastoma. U87 human malignant glioma cells that express an autocrine SF/HGF stimulatory loop were transfected with the human HGF/NK2 cDNA and clonal cell lines that secrete high levels of HGF/NK2 protein (U87-NK2) were isolated. The effects of HGF/NK2 gene transfer on the U87 malignant phenotype were examined. HGF/NK2 gene transfer had no effect on 2-dimensional anchorage-dependent cell growth. In contrast, U87-NK2 cell lines were ~20-fold less clonogenic in soft agar and ~4-fold less migratory than control-transfected cell lines. Intracranial tumor xenografts derived from U87-NK2 cells grew much slower than controls. U87-NK2 tumors were ~50-fold smaller than controls at 21 days post-implantation and HGF/NK2 gene transfer resulted in a trend toward diminished tumorigenicity. This report shows that the predominant effect of transgenic HGF/NK2 overexpression by glioma cells that are autocrine for SF/HGF stimulation is to inhibit their malignant phenotype. © 2000 Academic Press

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Scatter factor/hepatocyte growth factor (SF/HGF) is a multifunctional morphogen, motogen, and growth factor expression of which increases with tumor grade and correlates with tumor-associated angiogenesis in human glioma specimens (1, 2). Expression of the SF/HGF receptor c-met has also been found to correlate with the malignancy of human gliomas (3). Purified SF/HGF increases the proliferation and migration of a variety of cell types, including glioma and endothelial cells. Experimental intracerebral gliomas grow faster and display enhanced angiogenesis following SF/HGF gene transfer (4, 5). Furthermore, we have recently shown that the malignancy of experimental human gliomas can be dependent upon endogenous autocrine SF/HGF stimulatory loops (6). Thus, secreted SF/HGF antagonists might be attractive candidates for use in the biologic therapy of these aggressive neoplasms (7).

HGF/NK2 is a naturally occuring 28 kDa truncated form of SF/HGF derived from a 1.3 kb alternately spliced SF/HGF transcript. HGF/NK2 is comprised of the receptor-binding amino terminal end of SF/HGF including the first and second kringle domains. Secreted HGF/NK2 binds the SF/HGF receptor, c-met, with high affinity and functions as either a receptor antagonist or partial agonist. HGF/NK2 has been shown to lack endogenous mitogenic activity and to effectively inhibit SF/HGF-induced mitogenesis in mammary epthelial cells (8, 9) but alternatively to induce epithelial cell motility under other conditions *in vitro* (10, 11). HGF/NK2 was recently found to antagonize SF/HGF-dependent melanoma growth but to enhance SF/HGF-dependent melanoma metastasis *in vivo* (12). The effects of HGF/NK2 or other truncated forms of SF/HGF on glioma cell behavior have not been examined. In this report, we show that HGF/NK2 gene transfer to U87 MG human malignant glioma cells, a cell line previously shown to express an autocrine SF/HGF:c-met stimulatory loop (6), markedly inhibits their clonogenicity and migration *in vitro* and intracranial tumor growth *in vivo.*

MATERIALS AND METHODS

Cell culture. U87MG glioblastoma cells were cultured in Minimum Essential Medium (MEM) containing 15% FBS, 1 mM MEM sodium pyruvate, 0.1 mM MEM non-essential amino acids (GIBCO

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BRL, Grand Island, NY), and 1 μg/mL G418 (Mediatech, Inc, Herndon, VA). All cells were cultured at 37°C in 5% CO_2/95% air.

Plasmid construction and cell transfections. Full-length human HGF/NK2 cDNA (kindly provided by Drs. Jeff Rubin and Donald Bottaro; National Institutes of Health, Bethesda, MD) was inserted into the *Bam*HI restriction site immediately downstream from the MSV-LTR promoter of the eukaryotic expression vector pMEXneo, which also expresses the gene for neomycin resistance (13). This plasmid was designated pMEXneo-NK2. U87MG malignant glioma cells were transfected with pMEXneo-NK2 or with pMEXneo as a control, using the polycationic reagent Lipofectamine (15 μg/mL; GIBCO BRL). Transfected clonal cell lines were selected in the presence of 600 ng/mL G418 and designated U87-NK2 or U87-Control, respectively. Conditioned medium obtained from confluent cell lines was assayed for HGF/NK2 by immunoblot analysis using a monoclonal mouse anti-human SF/HGF antibody (provided by Dr. Jeff Rubin) specific for the amino terminus of both full length SF/HGF and NK2 proteins. Cell lines found to secrete high levels of NK2 were selected for further study.

Quantitative immunoblot assay. Samples of conditioned medium were collected in a standardized fashion from subconfluent monolayers of each neomycin-resistant cell line by incubating 1 mL serum-free MEM per well of 6-well plates for 24 h. Conditioned media were concentrated approximately 10-fold using Centricon YM-10 Centrifugal Filter Devices (Millipore; Bedford, MA) that retain all proteins of >10 kDa size. Concentrated conditioned media were assayed for total protein using the method of Bradford (Bio-Rad; Hercules, CA). Aliquots of conditioned medium containing 50 μg total protein were combined with 4× non-reducing Laemmli sample buffer and subjected to polyacrylamide gel electrophoresis at 50 mA under nonreducing conditions using 10% polyacrylamide gels (Bio-Rad). Proteins were electrophoretically transferred to nitrocellulose membranes (Amersham; Picastaway, NJ) in the presence of 20% methanol transfer buffer. Membranes were incubated in 1% Tris Borate Saline/0.1% Tween (TBST) containing 5% nonfat dried milk overnight, then washed with TBST. Membranes were then incubated with monoclonal mouse SF/HGF antibody described above in TBST containing 5% nonfat dried milk for 1 h, rinsed and then incubated with goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA) in TBST containing 5% nonfat dried milk for 1 h. Membranes were then washed, developed with ECL detection reagents (Amersham), and exposed to Hyper-film ECL (Amersham). Immunoreactive protein bands were quantified by densitometry using ImageQuant 3.22 software (Molecular Dynamics; Sunnyvale, CA). HGF/NK2 protein production per total protein for each individual U87-NK2 cell line was expressed relative to that found in U87-Control cell lines.

Cell proliferation assays. To assess anchorage-dependent proliferation, cells were added to 12-well plates (15,000 cells/well) in complete culture medium and incubated at 37°C in 5% CO_2/95% air. Cultures were then trypsinized at daily intervals ($n = 3$) and their numbers quantified using a Coulter Counter (Coulter, Inc., Hialeah, NY). Doubling times (DT) for each cell line during periods of logarithmic growth were determined using the formula DT = ln 2/(growth rate).

Anchorage-independent proliferation in soft agar was quantified as previously described, with minor modifications (5). Six-well culture plates were pre-coated with 1 mL MEM containing 1% FBS, 1% low melting agar, 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate (GIBCO BRL). After solidifying overnight, 10,000 cells suspended in 2 mL of medium containing 1% FBS, and 0.5% agar were added to each well. Cultures were incubated at 37°C in 5% CO_2/95% air and received fresh complete medium twice weekly. After approximately 2–3 weeks, cultures were fixed and stained with Wright's stain (Sigma; St. Louis, MO) and the number of colonies determined using video-based, computer-assisted image analysis.

Migration assay. Monolayer wound migration assays were performed as previously described with minor modifications (14). Cells (300,000) were plated into 60 mm tissue culture dishes containing 2 mm grid markings (Corning; Corning, NY) and incubated at 37°C in 5% CO_2/95% air. When approximately 90% confluent, all cells in a 20 mm wide section at the center of each plate were removed by scraping the cell monolayer with a sterile edge. Monolayers were then rinsed with PBS to remove all floating cells. Complete medium was added and the cells incubated for another 24–48 h after which they were rinsed with PBS and stained with Wright's stain. Cell migration was quantified by counting the number of cells that traveled into the 2 mm grid immediately adjacent to the wound. Twenty to forty randomly selected zones were quantified for each cell line.

In vivo tumor growth. Intracranial tumors were established and analyzed as previously described (6). U87-NK2 or U87-Control cells grown for at least one passage without G418 were trypsinized and resuspended in MEM (5×10^4 cells/ul) immediately before intracerebral implantation. Anesthesized 5–7 week-old SCID mice (Harlan; Gaithersburg, MD) received 2 μL of cell suspension into the right caudate/putamen by stereotactic injection (coordinates relative to bregma: 2.2 mm lateral, 2.5 mm deep) using a 26 guage needle. At 21 days post-implantation, mice were perfused with 4% paraformaldehyde. The brains were harvested, cryosectioned (20 μm thick), and stained with hematoxylin and eosin. As previously described, proportionate tumor volumes were calculated from measurements of maximal tumor cross-sectional area (MTA) obtained using computer-assisted image analysis, where volume is proportional to (square root of MTA)^3 (15).

Statistical analysis. Data on doubling times of clonal cell lines, *in vitro* anchorage-independent colony formation, migration, and *in vivo* tumor volume were analyzed using ANOVA followed by Bonferroni/Dunn multiple comparison tests unless otherwise indicated. All tests were performed using Statview 4.0. Numerical data are expressed as mean ± standard error of the mean.

RESULTS

HGF/NK2 Expression Levels of Transfected Human Glioma Cell Lines

Previous reports from our laboratoary established that wild type U87 human malignant glioma cells express both SF/HGF and its receptor c-met which function together in an autocrine stimulatory SF/HGF:c-met loop (1, 6). Wild-type U87 cells were transfected with a plasmid expression vector containing full length human HGF/NK2 cDNA. Controls were generated by transfecting cells with the same plasmid lacking the HGF/NK2 cDNA. Neomycin-resistant control-transfected and HGF/NK2-transfected clonal cell lines were screened and then quantitatively analysed for HGF/NK2 expression by immunoblot analysis of conditioned medium normalized to total protein. Several cell lines were identified with increased secretion of the expected 28 kDa HGF/NK2 protein at levels 6-fold to 350-fold higher than the very low levels of endogenous HGF/NK2 found in wild-type and control-transfected cell lines (Table 1 and Fig. 1). HGF/NK2 gene transfer had no apparent effect on the expression of endogenous SF/HGF in the transfected U87 cell lines examined (Fig. 1).

TABLE 1

HGF/NK2 Secretion by U87-NK2 Glioma Lines

U87-NK2 line	Relative NK2 secretion*
N16	10.5 × control
N23	6.0 × control
N34	290 × control
N36	346 × control

Note. Conditioned medium from control-transfected and HGF/NK2-transfected U87 cell lines were subjected to Western blot analysis using anti-HGF/NK2 antibodies as described under Materials and Methods. The HGF/NK2-specific band was quantified by computer densitometry for each cell line. HGF/NK2 secretion by each HGF/NK2-transfected U87 cell line (N16, N23, N34, and N36) relative to total secreted protein is expressed as the mean of triplicate determinations relative to the secretion of endogenous HGF/NK2 by two control-transfected U87 cell lines (C8 and C19).

* relative to control-transfected cell lines.

TABLE 2

Growth Rates of Control- and HGF/NK2-Transfected U87 Glioma Lines

Glioma line	Doubling time in hours*
C14	24.1 (1.6)
C19	24.2 (2.8)
N16	24.1 (3.3)
N23	29.2 (2.0)
N34	26.6 (0.9)

Note. Control-transfected (C14 and C19) and HGF/NK2-transfected (N16, N23, N34) U87 cells were inoculated at equal numbers per well in 24-well plates and incubated at 37°C in complete medium as described under Materials and Methods. Cell numbers were quantified daily in triplicate plates by Coulter counting. Doubling times (DT) during exponential growth were calculated using the formula DT = ln 2/(growth rate). No differences in growth rates between control- and HGF/NK2-transfected cell lines were observed.

* mean (sem).

Effects of HGF/NK2 Gene Transfer on Glioma Cell Proliferation in Vitro

The effect of transgenic HGF/NK2 expression on standard 2-dimensional anchorage-dependent cell growth was examined. Quantitation of doubling times during the exponential growth phase of U87-Control and U87-NK2 cell lines showed no significant effect of HGF/NK2 expression on glioma cell proliferation under these conditions (Table 2). The effect of HGF/NK2 expression on 3-dimensional anchorage-independent colony formation, an *in vitro* correlate of tumor cell maligancy, was also examined. As shown in Fig. 2, each U87-NK2 cell line examined was significantly less clonogenic than each U87-Control cell line when individually compared ($P < 0.001$). In addition, a comparison of pooled U87-Control cell lines with pooled U87-NK2 lines, also showed a substantial reduction in clonogenicity following HGF/NK2 gene transfer (82 ± 7.6 vs 4 ± 0.7 colonies/field, $P < 0.001$).

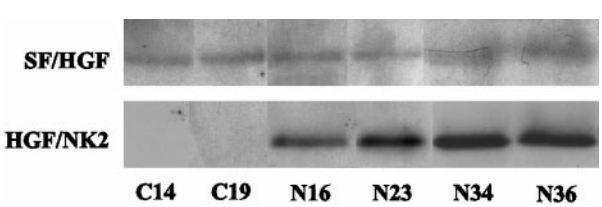

FIG. 1. HGF/NK2 expression by transfected cell lines. Conditioned medium collected from control-transfected (C14 and C19) and HGF/NK2-transfected (N16, N23, N34, N36) U87 glioma cell lines were subjected to immunoblot analysis under non-reducing conditions using SF/HGF antibody that recognizes both full length SF/HGF and HGF/NK2 as described under Materials and Methods. Endogenous full length SF/HGF (190 kDa) is produced by all cell lines. A prominent 28 kDa band corresponding to HGF/NK2 is readily detected in HGF/NK2-transfected cell lines only.

Effect of HGF/NK2 Gene Transfer on Glioma Cell Migration in Vitro

Since SF/HGF stimulates the motility of multiple cell types including human malignant glioma cell lines (2, 16–18), the effect of transgenic HGF/NK2 expression on human glioma migration was assessed using a standard cell monolayer wound assay. Each U87-NK2 cell line was signficantly less migratory when compared to each control cell line ($P < 0.001$) (Fig. 3). A comparison of pooled U87-Control cell lines with pooled U87-NK2 lines likewise revealed significantly diminished migratory indices following HGF/NK2 gene transfer (198 ± 9.3 vs 52 ± 3.8, $P < 0.001$).

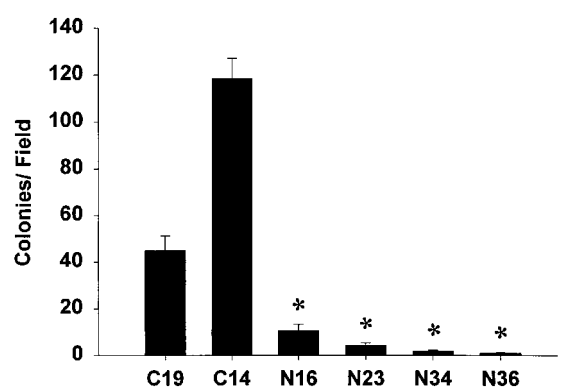

FIG. 2. HGF/NK2 gene transfer inhibits glioma cell clonogenicity in soft agar. Control-transfected (C14 and C19) and HGF/NK2-transfected U87 cells were suspended in 1% agarose, 1% FBS and added to each well of 6-well plates (10,000 cells/well) and incubated at 37°C for 3 weeks as described under Materials and Methods. Wells were fixed and stained with Wright's stain and colonies analyzed by computer-assisted image analysis. The number of colonies >125 μm diameter were counted per field. Data represents the mean ± SEM of 4 microscopic fields per well and 6 wells per cell line. * $P < 0.001$ compared to either control cell line.

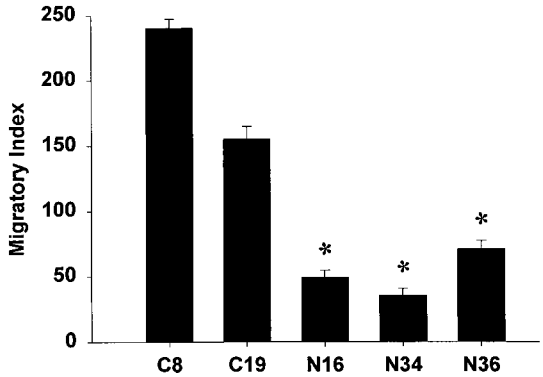

FIG. 3. NK2 expression inhibits glioma cell migration. Control-transfected and HGF/NK2-transfected U87 cell lines were plated at 300,000 cells per well in 60 mm diameter gridded tissue culture dishes. When cells achieved 90% confluency, a 20 mm × 40 mm "wound" was cleared of cells after which the plates were washed and replaced with medium. After 24 h, plates were fixed and stained with Wright's stain. The number of cells that entered the wound were counted under phase-contrast microscopy and expressed as the Migratory Index. Data represents mean ± SEM. * indicates $P < 0.001$ compared to either control.

TABLE 3

Effect of HGF/NK2 Gene Transfer on Growth of U87 Glioma Xenografts

Glioma line	Tumor volume (mm^3)
C8	10.6 (2.7)
C19	108.3 (45.9)
Pooled U87-Control	59.4 (25.1)
N16	0.92 (0.26)*
N23	0.10 (0.07)*
N34	0.01 (0.01)*
N36	2.79 (0.95)*
Pooled U87-NK2	1.09 (0.35)*

Note. HGF/NK2-transfected (U87-NK2) and control-transfected (U87-Control) cell lines were stereotactically injected into the caudate putamen of SCID mice (100,000 cells per brain). Brains were perfused and removed at 3 weeks post-implantation. Cryostat sections (20 μm thick) were stained with hematoxylin and eosin and maximum cross-sectional areas were measured by computer-assisted image analysis. Data represents mean (standard error of the mean), *$P < .001$ in comparison to pooled U87-Control tumors by Bonferroni/Dunn test.

Effect of HGF/NK2 Gene Transfer on Intracranial Glioma Growth

We have previously found that autocrine and paracrine SF/HGF:c-met signaling enhances the growth, tumorigenicity, and tumor-associated angiogenesis of experimental rat and human gliomas *in vivo* (3, 4). Therefore, the effects of HGF/NK2 expression on intracranial U87 xenograft growth were examined. U87-NK2 and U87-Control cell lines were implanted stereotactically into the caudate/putamen of immune deficient mice. After 21 days, animals were sacrificed and brain sections were analyzed for tumor formation and tumor size.

The intracranial tumors resulting from U87-NK2 cell lines were exceedingly small in comparison to those resulting from the U87-Control cell lines (Table 3 and Fig. 4). A comparison of pooled U87-NK2 tumors with pooled U87-Control tumors revealed that HGF/NK2 gene delivery resulted in significantly diminished tumor growth as evidenced by a 50-fold reduction in mean tumor volumes (1.09 ± 0.38 vs 59.4 ± 25 mm^3, $P < 0.03$). There was also a trend of diminished tumorigenicity following HGF/NK2 gene transfer. While all animals receiving U87-Control cells developed tumors (16 of 16 animals), only 22 of 27 animals (81%) implanted with U87-NK2 cells developed histologically-identifiable tumor masses ($P = 0.08$, Fisher's Exact Test).

DISCUSSION

Recent studies on human tumor specimens and in experimental tumor models have identified SF/HGF as an important determinant of malignancy in an array of solid tumors including gliomas (1, 3, 19–23). SF/HGF elicits a number of defined autocrine and paracrine responses involved in the stimulation of solid tumor growth and metastasis. These include: (i) mitogenicity in a variety of cell types, including glioma and endothelial cells (2, 18), (ii) stimulation of glioma cell motility and invasion *in vitro* (2, 16–18), (iii) angiogenic activity in several *in vivo* models (5, 24–27), (iv) induction of proteolytic enzymes such as plasminogen activators and matrix metalloproteinases involved in tumor cell migration, invasion and angiogenesis (5, 28–30), and (v) reduction of apoptotic responses to cytotoxic agents (31). The SF/HGF cell surface receptor is the proto-oncogene c-met tyrosine kinase capable of activating multiple second messenger pathways in-

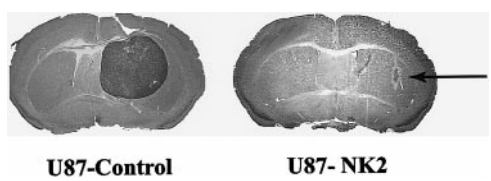

FIG. 4. HGF/NK2 gene transfer inhibits growth of human glioma xenografts *in vivo.* Representative histological sections of brains bearing U87-NK2 and U87-Control tumor xenografts stained with hemotoxylin/eosin. Control-transfected and HGF/NK2-transfected U87 glioma cells were stereotactically implanted (100,000 cells/brain) to the caudate putamen of immunodeficient SCID mice. The brains were perfusion-fixed and removed 3 weeks post-implantation. Cryostat sections (20 μm thick) were stained with hematoxylin and eosin. Tumor volumes were calculated from maximal tumor cross-sectional area (MTA) measurements using the formula volume = (square root of MTA)^3 (see Table 3).

cluding MAP-kinase, PI3-kinase/AKT, and STAT-3 that are linked to developmental events and malignancy (32). Both SF/HGF and/or c-met expression are clinically associated with poor prognosis in several human carcinomas (33–40), and expression levels correlate with malignant grade in the human gliomas (2, 3, 41, 42). Furthermore, SF/HGF gene transfer enhances experimental glioma growth, tumorigenicity, tumor-associated angiogenesis, and blood-tumor barrier dysfunction consistent with a role in the malignant progression of these tumors (4, 5, 43).

Inhibiting the SF/HGF:c-met pathway can effectively reverse the malignant phenotype of experimental human gliomas (6). Inhibition of either endogenous SF/HGF or c-met expression in U87 glioma cells that express an endogeneous autocrine SF/HGF:c-met stimulatory loop using chimeric ribozymes significantly reduces *in vitro* U87 clonogenicity, *in vivo* tumorigenicity, and *in vivo* tumor growth following the intracranial implantation of stably transfected glioma cells (6). Thus human glioma growth can be dependent upon endogenous SF/HGF and/or c-met expression. Since standard glioma treatments have minimal efficacy, targeting a multifunctional growth factor system such as the SF/HGF:c-met pathway offers a promising new treatment strategy (44). Since ribozyme-based strategies require highly efficient gene targeting of the majority of cells comprising a large tumor, applications requiring *in vivo* gene transfer may be limited by current inefficiencies of gene delivery technology. Identifying purified proteins or transgene products capable of antagonizing the stimulatory effects of SF/HGF on human gliomas is of interest.

SF/HGF is translated as a single polypeptide that is cleaved to form a functional heterodimer consisting of a 69 kDa alpha-chain and a 34 kDa beta-chain linked via disulfide bonds (45). Structural/functional analyses have determined that amino terminal sequences extending to the first and second kringle domains of the alpha chain mediate SF/HGF binding activity to its receptor c-met. Two naturally occuring truncated variants of SF/HGF consisting of the amino terminal sequences extending through the first and second kringle domains have been identified and designated HGF/NK1 and HGF/NK2, respectively (8, 9, 46). The biological properties of HGF/NK1 and HGF/NK2 have been examined extensively in epithelial cell lines. Both HGF/NK1 and HGF/NK2 effectively compete with full length heterodimeric SF/HGF for receptor binding. HGF/NK1 retains substantial mitogenic and migration stimulatory activity (46, 47). In contrast HGF/NK2 is relatively defective in stimulating c-met receptor activation and inhibits the ability of intact SF/HGF to stimulate c-met receptor autophosphorylation and subsequent DNA replication (8, 12). In many systems HGF/NK2 competitively inhibits the mitogenic, scattering, and angiogenic activities of full-length SF/HGF *in vitro* (10, 30, 48, 49). However, some studies have demonstrated a low potency partial agonist activity of HGF/NK2 in epithelial cells that varies with local heparin concentrations (47), and HGF/NK2 appears to retain the ability to stimulate the migration of some epithelial cell lines *in vitro* (10, 11). Consistent with these *in vitro* studies are the recent findings of Otsuka *et al.* who elegantly show that NK2 antagonizes most of the pathologic effects of SF/HGF overexpression on organ development and tumor growth with the exception of tumor metastasis using bitransgenic mouse models (12). In addition to these natural SF/HGF fragments, Date *et al.* generated an artificial proteolytic fragment of the SF/HGF alpha chain composed of the amino terminal end and all four kringle domains (HGF/NK4) that has no agonist properties and effectively competes with SF/HGF receptor activation (50). These investigators subsequently used systemic HGF/NK4 delivery to inhibit the growth of subcutaneous bladder carcinoma xenografts.

In this report we explore the potential of HGF/NK2 gene transfer to reverse the malignant phenotype of the U87 glioma cell line that expresses an autocrine SF/HGF:c-met loop that augments clonogenicity *in vitro* and tumor growth *in vivo* (6). We show that HGF/NK2 gene transfer inhibits U87 clonogenicity and migration *in vitro* and tumor growth *in vivo*—malignant properties previously found to be enhanced in gliomas by either purified or transgenic native SF/HGF. This is the first use of HGF/NK2 to inhibit a malignant phenotype in human CNS cells and is consistent with the findings of Otsuka *et al.* and Date *et al.* using systemic melanoma and carcinoma tumor models, respectively, as described above. The ability of HGF/NK2 gene transfer to inhibit glioma cell clonogenicity and migration *in vitro* is consistent with the antagonism of the endogenous SF/HGF:c-met loop that is active in the U87 glioma cell line. Based on our previous finding that SF/HGF gene transfer enhances intracranial glioma angiogenesis and that of Lamszus *et al.* (2) demonstrating that SF/HGF levels in human tumor specimens correlate with vessel density, it is possible that U87 secretion of transgenic HGF/NK2 might also inhibit tumor growth by inhibiting the paracrine effects of endogenous tumor-derived SF/HGF on glioma angiogenesis. Unfortunately, the small size of tumors following HGF/NK2 gene transfer precluded the reliable quantification of an anti-angiogenic effect in this study. SF/HGF antagonists such as HGF/NK2 and HGF/NK4 also have the potential to sensitize glioma cells to cytotoxic treatment modalities such as chemotherapy and radiation therapy through their potential antiangiogenic action or their ability to interfere with the direct cytoprotective actions of SF/HGF on tumor cells (31). The ability of SF/HGF antagonists to interfer with multiple pathophysiologic processes associated with glioma malignancy make them particularly suited to

therapeutic applications including direct intratumoral gene-delivery, intratumoral convection-based protein delivery, and systemic protein-based strategies.

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