Inhibition of Intracerebral Glioblastoma Growth by Local Treatment with the Scatter Factor/Hepatocyte Growth Factor-Antagonist NK4

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ABSTRACT

Purpose: **Scatter factor/hepatocyte growth factor (SF/HGF) and its tyrosine kinase receptor MET are strongly up-regulated in malignant gliomas. The SF/HGF-MET system contributes to glioma invasion and angiogenesis via autocrine and paracrine mechanisms. We analyzed whether local treatment with NK4, an antagonistic fragment of SF/HGF, could inhibit glioma growth *in vivo*.**

Experimental Design: **A guide-screw system was used to implant tumor cells intracerebrally and to perform therapeutic injections. Mice received daily intratumoral injections of NK4 or buffer as of day 1 or 7 after tumor cell injection until day 20. Functional effects of NK4 on glioma and endothelial cells were analyzed *in vitro*.**

Results: **Tumor volume was reduced by 61.1% in mice treated with NK4 compared with controls when treatment was initiated on day 1 ($P < 0.05$) and by 61.4% when treatment was initiated on day 7 ($P < 0.001$). Intratumoral microvessel density was reduced by 64.9% when treatment started on day 1 and by 36.7% when it started on day 7. The proliferative activity of the tumor cells was reduced by >30% regardless of when NK4-treatment was initiated. The apoptotic fraction of tumor cells was increased 2-fold and 1.5-fold when animals were treated with NK4 as of day 1 or day 7, respectively. *In vitro*, NK4 inhibited SF/HGF-induced glioblastoma, and endothelial cell migration and proliferation in a dose-dependent fashion.**

Conclusion: **NK4 inhibits glioblastoma growth *in vivo*, most likely via antimitogenic, antimotogenic, proapoptotic, and antiangiogenic mechanisms. Given the strong up-regulation of SF/HGF and MET in human malignant gliomas, NK4 holds promise as a direct interstitial therapeutic agent for these fatal tumors.**

INTRODUCTION

Despite aggressive therapeutic approaches combining surgical resection, radiotherapy, and chemotherapy, the mean survival time of patients diagnosed with glioblastoma is only ~1 year. Novel strategies are needed to treat these fatal tumors, which also represent the most common type of brain neoplasm. Glioblastomas are highly vascularized and depend on the formation of new blood vessels; therefore, antiangiogenic therapies are considered promising. However, glial neoplasms not only grow as solid tumor foci but also spread diffusely throughout the brain. The brain itself has a dense vascular bed, and gliomas preferentially invade along these vessels, a process that was recently termed "vessel cooption" (1). We and others showed that inhibition of angiogenesis in an orthotopic glioblastoma model could strongly inhibit growth of the main tumor mass, but, in turn, favored glioma cell invasion along preexistent host vessels (2, 3). Therefore, therapeutic strategies that synchronously inhibit tumor angiogenesis, as well as tumor cell migration and proliferation appear to hold greater promise than a mere antiangiogenic approach.

SF/HGF[2] is a pleiotropic factor that simultaneously induces tumor and endothelial cell migration and proliferation, as well as angiogenesis *in vivo* (4). The SF/HGF molecule is a heterodimer composed of a 69-kDa α chain containing an NH_2-terminal hairpin domain and four kringle domains, linked by a disulfide bridge to a 34-kDa serine protease-like β chain. The SF/HGF-receptor MET is a transmembrane tyrosine kinase encoded by the *c-met* proto-oncogene. Both SF/HGF and MET are expressed in gliomas, and are associated with increased malignancy grade and vascularity (5, 6). Functionally, SF/HGF expression promotes glioblastoma growth *in vivo* (7), and stimulates glioma and cerebral microvascular endothelial cell motility and proliferation *in vitro* (8, 9).

NK4 is a synthetic molecule that comprises the NH_2-terminal hairpin domain and subsequent four kringle domains of SF/HGF, but lacks the entire β chain. NK4 competes with SF/HGF for the MET receptor and, thus, inhibits SF/HGF-induced effects on tumor and endothelial cells (10, 11). It

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[2] The abbreviations used are SF/HGF, scatter factor/hepatocyte growth factor; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; vWF, von Willebrandt factor; hpf, high power field.

inhibits the specific binding of SF/HGF to its receptor MET in a competitive fashion. Because of its combined antiangiogenic, anti-invasive, and antiproliferative effects, NK4 offers a promising approach, especially for glioblastoma therapy.

In the present study, we analyzed whether NK4 could inhibit glioblastoma growth in an orthotopic nude mouse model. We used a guide-screw system that was developed recently to facilitate repeated precise intratumoral injections into intracerebral tumor xenografts in mice (12). To study the mechanisms of NK4-mediated tumor inhibition, we analyzed its effects on glioblastoma and endothelial cells *in vitro*.

MATERIALS AND METHODS

Cell Culture and Preparation of Conditioned Medium. The human U-87 MG glioblastoma cell line was cultured in DMEM (Life Technologies, Inc., Paisley, Scotland) supplemented with 10% FBS. To generate conditioned medium, subconfluent cultures were incubated in serum-free medium consisting of equal volumes of Ham's F12 (Biochrom, Berlin, Germany) and DMEM with the addition of 10 μg/ml insulin, 10 μg/ml transferrin, 10^{-8} M selenium, 1% BSA, and 1 mg/ml linoleic acid (all from Sigma, St. Louis, MO) for 48 h. The medium was centrifuged to remove cellular debris and concentrated 24-fold using Centriprep concentrators (Millipore, Bedford, MA).

HUVEC isolation from freshly obtained human umbilical cords was performed as described previously (13). Cells were grown on 1% collagen I-coated plasticware in M199 medium (Life Technologies, Inc.) supplemented with 20% FBS, 90 μg/ml endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (all from Life Technologies, Inc.), and 40 units/ml heparin. HUVECs were used for experiments between passages 5 and 8.

Preparation of NK4. NK4 was produced recombinantly in HEK293 cells and subsequently purified by column chromatography. Briefly, an expression plasmid coding for NK4 was constructed and transfected into the human embryonic kidney cell line 293. Positive transfectants were selected with G418, and cells were cloned. The best clone was used to perform 10-liter or 100-liter fermentations. Culture supernatant was applied onto heparin Sepharose (Pharmacia, Erlangen, Germany), and bound protein was eluted with a linear sodium chloride gradient at pH 8. NK4-containing fractions were additionally purified by ion exchange chromatography on S-Sepharose (Pharmacia). The final preparation was >95% pure according to reversed-phase high-performance liquid chromatography and SDS-PAGE analysis.

Orthotopic Glioma Model. Female 6–8-week-old nude mice (NMRI-*nu/nu*) were used. Institutional guidelines for animal welfare and experimental conduct were followed. A guide-screw system (Plastics One Inc., Roanoke, VA) was used to perform the intracerebral tumor cell engraftment and subsequent intratumoral injections of recombinant human NK4 (provided by Roche Diagnostics, Penzberg, Germany) or buffer. Guide-screws were implanted after a protocol described by Lal *et al.* (12) with some modifications. Mice were anesthetized by i.p. administration of ketamine (100 mg/kg body weight) and xylazine (5 mg/kg body weight). A piece of the scalp and of the underlying periost was removed, and a 1-mm burrhole was drilled into the skull 3.5 mm lateral to the bregma. The guide-screw was screwed into the burrhole using a special screwdriver (Plastics One). Two-component adhesive (UHU GmbH, Buehl, Germany) was used to glue the guide-screw to the skull. The central hole of the guide-screw was occluded using a magnetic stylet provided with the screw. Three days after the implantation of guide-screws, 1.5×10^6 U-87 MG cells were injected through the screw into the basal ganglia of the mice using a 26-gauge needle attached to a 25 μl Hamilton syringe. For the injection of test substances, mice were anesthetized as described above, the stylet was removed, and NK4 (25 μg/day in 2.5 μl of 100 mM $NaPO_4$ buffer solution; pH 7.2) or buffer solution (2.5 μl 100 mM $NaPO_4$) was injected over 2 min. Afterward, freshly sterilized stylets were inserted. Treatment was initiated on day 1 after tumor cell implantation and was continued daily over 3 weeks. The experiment was repeated with a modified schedule in which mice received NK4 and buffer in the same doses, but treatment was initiated on day 7 and mice were treated over only 2 weeks. Three weeks after tumor cell implantation, all of the mice were killed using CO_2.

Determination of Tumor Size. Mouse brains were removed from the cranial cavity, fixed in formalin overnight, bisected coronally, and embedded in paraffin. Serial sections (5 μm thick) were stained with H&E. The maximum cross-sectional area of the intracranial glioblastoma xenografts was determined by computer-assisted image analysis using a Leica Quantimet 500-system (Leica, Hamburg, Germany). The tumor volume was estimated using the formula: volume = (square root of maximal tumor cross-sectional area)^3.

Determination of Microvessel Density, Proliferative Activity, and Apoptosis. For immunohistochemistry, paraffin sections were dewaxed using standard histological procedures. To stain microvessels, sections were incubated with an antibody against vWF (1:400) for 1 h at room temperature, and bound antibody was detected using the Vectastain kit (Vector Laboratories, Burlingame, CA). Vessel density was determined by counting the number of stained vessels in 3 hpfs (0.031 mm^2) that were either selected in the most densely vascularized "hot spot" area or randomly approximately in the center of the tumor avoiding necrotic areas.

To analyze the proliferative activity of the tumor cells, sections were stained with an antibody against the Ki-67 antigen using the Vectastain kit. The percentage of MIB-1-positive nuclei was determined by counting immunoreactive tumor cell nuclei in 3 randomly selected hpfs, as well as in 3 hpfs in the most actively proliferating tumor area ("hot spot").

To detect apoptotic cells, paraffin sections were stained using an antibody that specifically binds to single-stranded DNA of apoptotic cells (Apostain; Alexis Biochemicals, San Diego, CA), following the manufacturer's instructions. The sections were pretreated with proteinase K (20 μg/ml). Detection of bound Apostain antibody was performed using the Vectastain kit. The percentage of apoptotic cells was determined by counting stained tumor cell nuclei in 3 randomly selected hpfs.

Cell Proliferation Assays. HUVECs were seeded into collagen-coated 96-well plates (2000 cells/well) and cultured overnight. On day 1, cells were washed once with PBS, and the

medium was replaced with endothelial cell basal medium (EBM-2; Clonetics, Wakerville, MD) containing 1% FBS, 25 μg/ml ascorbic acid, 5 ng/ml insulin-like growth factor 1, 20 units/ml heparin, and 0.2 μg/ml cortisol, as well as SF/HGF (Genentech Inc., South San Francisco, CA) and NK4. Medium and test substances were renewed on day 4. Quadruplicate wells were fixed at daily intervals using 1% glutaraldehyde. After 7 days, fixed cells were stained with crystal violet, washed with PBS, solubilized in 10% SDS, and the absorbance of the lysate was quantified by reading the absorbance at 540 nm (Bio-Tek Instruments, Winooski, VT).

U-87 MG cells did not tolerate low serum conditions in 96-well plates >7 days, therefore, a different proliferation assay was used. Cells were plated onto 48-well plates (6500 cells/well) and cultured for 24 h. After washing with PBS, the medium was replaced by serum-free medium with and without SF/HGF and NK4, which was renewed after 4 days. On days 4 and 6 of the experiment, the cells were trypsinized and counted.

Modified Boyden Chamber Migration Assay. The effect of SF/HGF, NK4, and U-87 MG-conditioned medium on the motility of U-87 MG cells and HUVECs was analyzed using a modified Boyden chamber migration assay as described previously (8). Briefly, SF/HGF and NK4 were diluted in serum-free medium and added to the lower wells of a 96-well modified Boyden chamber (Neuroprobe, Cabin John, MD). Wells were covered with an 8-μm pore size Nucleopore filter (Neuroprobe) coated with Vitrogen 100 (Cohesion, Palo Alto, CA). Triplicates of U-87 MG cells were seeded into the upper wells at 1.5×10^4 cells/well in 50 μl serum-free M199 medium containing 0.1% BSA. After incubation at 37°C for 5 h, nonmigrated cells were scraped off the upper side of the filter, and filters were stained with Diff-Quick (Dade, Unterschleissheim, Germany). Nuclei of migrated cells were counted in 10 high-power fields using a ×40 objective.

SF/HGF ELISA. The concentration of SF/HGF in the conditioned medium collected from U-87 MG cells was determined using a commercial ELISA kit (R&D Systems, Minneapolis, MN).

Statistics. Differences between the NK4 treatment and the control group, as well as differences in the *in vitro* experiments, were analyzed using the unpaired *t* test or Mann-Whitney rank-sum test. P values < 0.05 were considered to be statistically significant.

RESULTS

Tumor Growth *in Vivo*. The U-87 MG cell line secretes SF/HGF and expresses the MET receptor, and was therefore chosen for *in vivo* experiments. The cells were implanted through a guide-screw into the caudate/putamen of nude mice on day 0, and intratumoral treatment with NK4 or buffer solution was initiated on day 1 after tumor cell injection. Treatment was continued daily over 3 weeks. Compared with the control group, the mean tumor volume in mice treated with NK4 was reduced by 61.1% (6.8 ± 4.9 mm^3 *versus* 17.5 ± 10.7 mm^3; $P < 0.05$; Fig. 1*A*; Fig. 2, *A* and *B*). Tumor sizes ranged from 1.9 to 17.3 mm^3 in the NK4-treated animals, and from 7.5 to 34.7 mm^3 in controls. In a modified repeat experiment, treatment was initiated on day 7 after tumor cell injection and continued over 2 weeks to investigate whether NK4 treatment was also effective against already-established tumors. By the end of this second experiment, the mean tumor volume in mice treated with NK4 was reduced by 61.4% compared with the control group (9.6 ± 5.7 mm^3 *versus* 24.9 ± 8.1 mm^3; $P < 0.001$; Fig. 1*A*). Tumor sizes ranged from 1.6 to 20.1 mm^3 in NK4-treated animals, and from 10.0 to 30.9 mm^3 in controls.

Tumor Microvessel Density. Intratumoral vessel densities were quantified after staining histological sections for vWF, which specifically labels endothelial cells. In NK4-treated mice, the mean intratumoral microvessel density, as counted in 3 randomly selected hpfs, was reduced by 64.9% relative to tumors in the control group ($P < 0.005$; Fig. 1*B*; Fig. 2, *C* and *D*). Similarly, when comparing the most densely vascularized "hot spot" areas of the tumors, vessel density was found to be reduced by 47.9% ($P < 0.01$; Fig. 1*C*). Initiation of treatment with NK4 7 days after xenograft implantation resulted in a reduction of the randomly counted mean intratumoral vessel density by 36.7% compared with controls ($P < 0.001$; Fig. 1*B*), whereas the maximum vessel density was reduced by 43.0% ($P < 0.001$; Fig. 1*C*). The vasculature in the surrounding normal brain was not affected by the treatment.

Tumor Cell Proliferation. Tumor cell proliferation was quantified on histological sections as the percentage of Ki-67-expressing nuclei (MIB-1 labeling index). Evaluating the mean proliferative activity in 3 randomly selected hpfs, the MIB-1 labeling index was found to be reduced by 33.0% in tumors of NK4-treated mice compared with controls ($P = 0.01$; Fig. 1*D*; Fig. 2, *E* and *F*). Similar results were obtained when analyzing areas of maximum cell proliferation; in this comparison, the MIB-1 labeling index was reduced by 25.6% in the NK4 treatment group ($P < 0.05$; Fig. 1*E*). When treatment was initiated on day 7, the mean proliferative index was reduced by 38.8% in the NK4-treated group compared with controls ($P < 0.001$; Fig. 1*D*), and the maximum proliferation index was reduced by 14.0% ($P < 0.05$; Fig. 1*E*).

Tumor Cell Apoptosis. The apoptotic index was quantified on histological sections as the percentage of Apostain-immunoreactive tumor cell nuclei. NK4-treated animals displayed a 2.0-fold higher percentage of apoptotic tumor cells in comparison with controls when treatment was initiated on day 1 ($P < 0.001$; Fig. 1*F*; Fig. 2, *G* and *H*). A tendency for an increased apoptosis rate was also observed when NK4 treatment was initiated on day 7; in this experiment, the fraction of apoptotic tumor cells was increased 1.5-fold compared with controls ($P = 0.06$; Fig. 1*F*). Between one third and half of the tumors in both treatment groups contained a central necrosis; however, we found no difference in the frequency or sizes of necroses between the two treatment groups in either experiment.

Tumor and Endothelial Cell Migration *in Vitro*. Effects of NK4 on glioma and endothelial cell motility were analyzed using a modified Boyden chamber migration assay. Chemotactic migration of U-87 MG cells was strongly induced by SF/HGF, and a maximum response of 21-fold stimulation was observed at 100 ng/ml (Fig. 3*A*). Stimulation of tumor cell motility by SF/HGF was dose-dependently inhibited by NK4, which at 10 μg/ml almost completely suppressed SF/HGF-induced migration up to a SF/HGF concentration of 200 ng/ml (Fig. 3*A*).

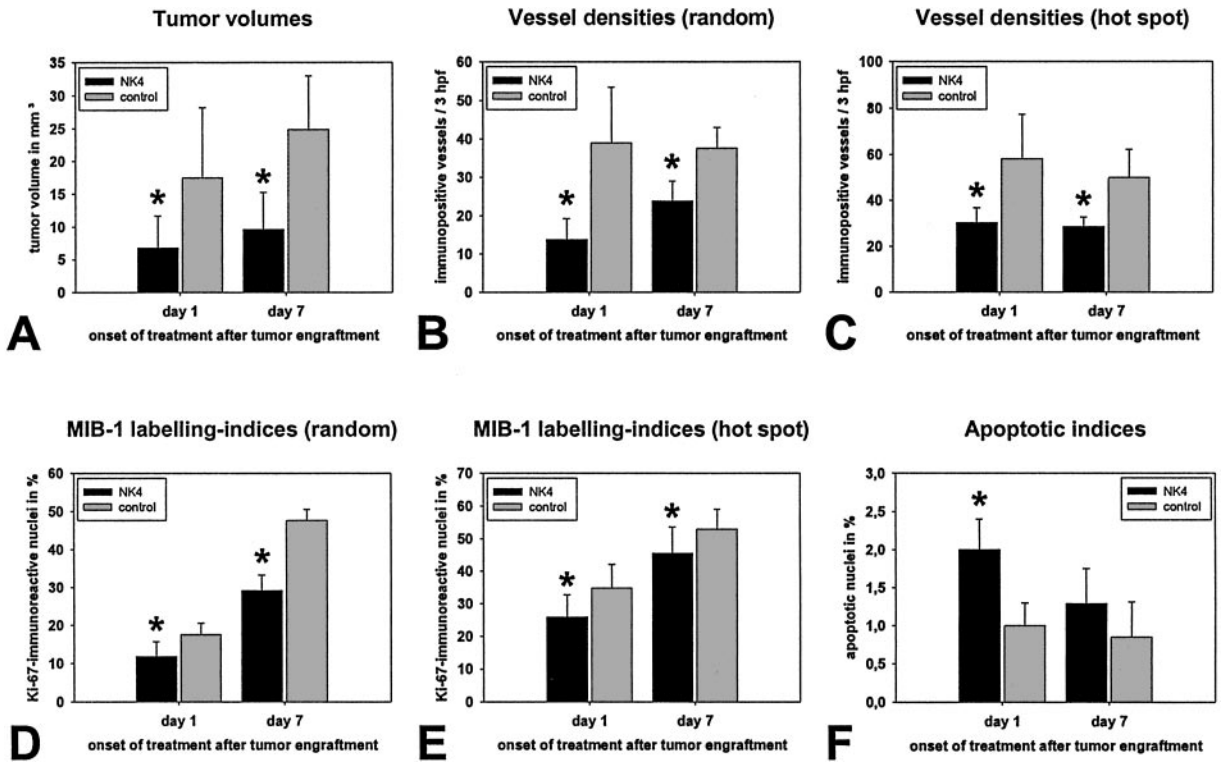

Fig. 1 Comparisons between tumors in NK4-treated mice and control mice. Treatment was initiated either on day 1 or on day 7 after tumor cell engraftment. *A,* tumor volume was reduced by 61.1% in mice treated with NK4 as of day 1 compared with controls, and by 61.4% in mice treated as of day 7. *B,* vessel densities in three randomly selected fields were reduced significantly in tumors from NK4-treated animals, and *C,* similar results were obtained when analyzing the most densely vascularized "hot spot" areas. *D,* the proliferative activity in three randomly selected fields and *E,* in the most actively proliferating tumor areas were reduced significantly in NK4-treated tumors compared with controls. *F,* the percentage of apoptotic tumor cells was increased in mice treated with NK4 as of day 1, whereas this difference did not quite reach significance when treatment was initiated on day 7. ∗ in *A–F* indicate significance; *Bars,* ±SD.

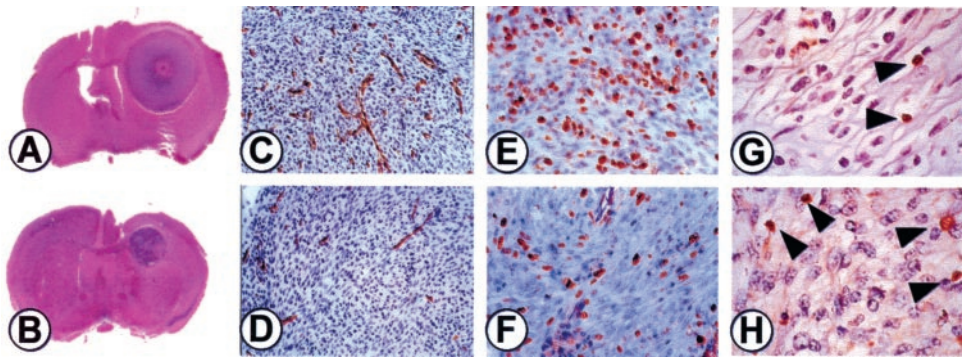

Fig. 2 Histological examination of tumors in NK4-treated mice and controls. *A,* representative size examples of U-87 MG-derived tumors grown intracerebrally in mice that received buffer or (*B*) NK4; paraffin sections were stained with H&E. *C,* immunostaining for vWF revealed numerous small capillaries in control tumors, but *D* only sparse vascularization in tumors from NK4-treated mice. *E,* the percentage of Ki-67 immunoreactive tumor cells was higher in control tumors than in (*F*) tumors from NK4-treated animals. *G,* the fraction of apoptotic tumor cells (*arrows* in *G* and *H*) as determined by Apostain immunoreactivity, was lower in tumors from control animals than (*H*) in tumors from NK4-treated mice.

Using a specific ELISA, we determined that 1×10^6 U-87 MG cells secreted ~0.5 ng SF/HGF/day into the medium. Autocrine stimulation of tumor cell motility was analyzed using concentrated conditioned medium prepared from U-87 MG cells, which contained 25 ng/ml SF/HGF. The medium strongly stimulated chemotactic migration of U-87 MG cells in a dose-dependent fashion with a maximally 39-fold effect at 40% conditioned medium (≅10 ng/ml SF/HGF; Fig. 3*B*). The motogenic effect of the conditioned medium was inhibited completely (up to 40% conditioned medium) by coaddition of 10

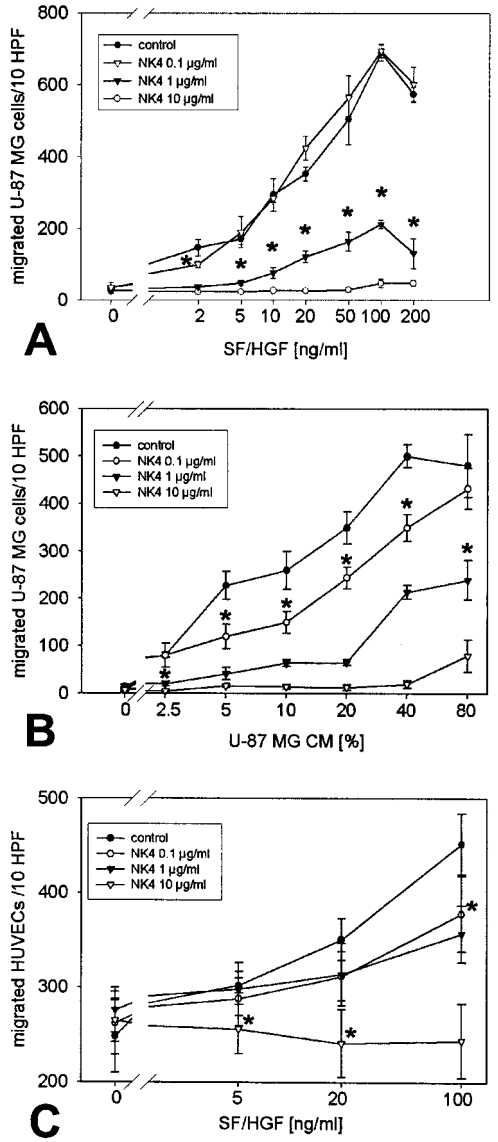

Fig. 3 Inhibition of SF/HGF-induced chemotactic migration of U-87 MG cells and HUVECs by NK4. *A,* SF/HGF was added to the lower wells of a modified Boyden chamber assay in the absence or presence of NK4; U-87 MG cells were seeded into the upper wells; significant inhibition of migration was obtained at 1 μg/ml NK4 for almost all SF/HGF concentrations analyzed. *B,* 24-fold concentrated conditioned medium (*CM*) collected from U-87 MG cells grown under serum-free conditions, was added at different percentages to the lower wells of the assay chamber with or without coaddition of NK4; U-87 MG cells were seeded into the upper wells. 0.1 μg/ml NK4 caused significant inhibition of CM-induced migration at most concentrations analyzed. *C,* SF/HGF with and without NK4 was added to the lower wells of the chamber, and HUVECs were seeded into the upper wells. Complete inhibition of migration was obtained at 10 μg/ml NK4. Values are means of triplicate determinations; *bars,* ±SD. ∗ in *A–C* mark the minimum concentration of NK4 required to obtain significant inhibition of migration.

μg/ml NK4, whereas lower concentrations were less effective (Fig. 3*B*).

We additionally analyzed the chemotactic effects of SF/HGF and NK4 on human endothelial cells. SF/HGF induced

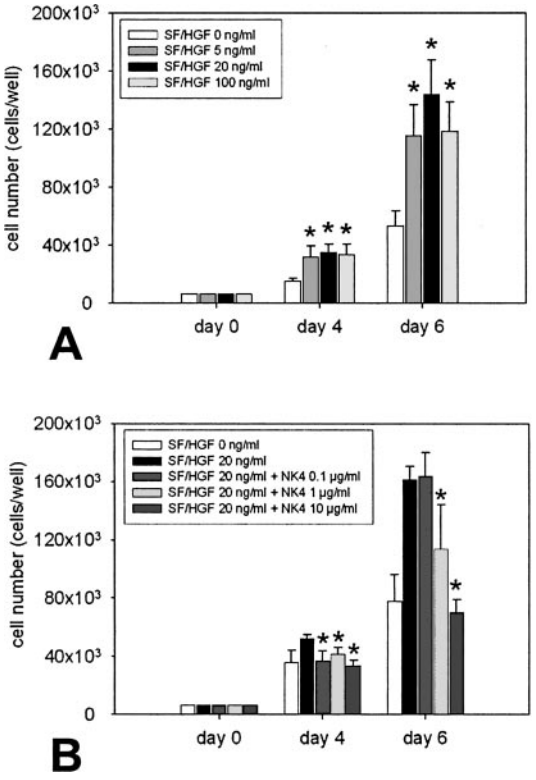

Fig. 4 Inhibition of SF/HGF-induced proliferation of U-87 MG cells by NK4. *A,* cell numbers were counted after 4 and 6 days. Maximum stimulation was obtained at 20 ng/ml SF/HGF. ∗ mark significant stimulation by SF/HGF. *B,* proliferation was stimulated by 20 ng/ml SF/HGF and was inhibited by coaddition of NK4. ∗ indicate significant inhibition of SF/HGF-induced proliferation by NK4. Values are means of six different determinations; *bars,* ±SD.

directional migration of HUVECs, albeit less strongly than of U-87 MG cells, with a maximum effect of 1.8-fold stimulation ($P < 0.005$) at 100 ng/ml. The effect was blocked completely by coaddition of 10 μg/ml NK4 (Fig. 3*C*).

Tumor and Endothelial Cell Proliferation *in Vitro*. Proliferation of U-87 MG cells in response to SF/HGF was analyzed after 4 and 6 days. Maximum stimulation of proliferation was observed at 20 ng/ml SF/HGF (2.7-fold stimulation on day 6; Fig. 4*A*). Therefore, this concentration was chosen for subsequent inhibition experiments with NK4. Until day 4, coaddition of NK4 even at low concentrations (≥0.1 μg/ml) completely inhibited SF/HGF-induced proliferation, whereas on day 6, significant inhibition was obtained only by concentrations ≥1 μg/ml NK4, and 10 μg/ml NK4 inhibited SF/HGF-induced proliferation completely (Fig. 4*B*).

Similar to U-87 MG cells, HUVEC proliferation was stimulated most strongly at 20 ng/ml SF/HGF, as analyzed over 7–8 days using a colorimetric assay (Fig. 5*A*). Coaddition of NK4 at 10 μg/ml completely inhibited SF/HGF-induced stimulation of proliferation (Fig. 5*B*).

DISCUSSION

Human glioblastoma cells strongly express SF/HGF and MET *in vivo*, whereas most established glioblastoma cell lines

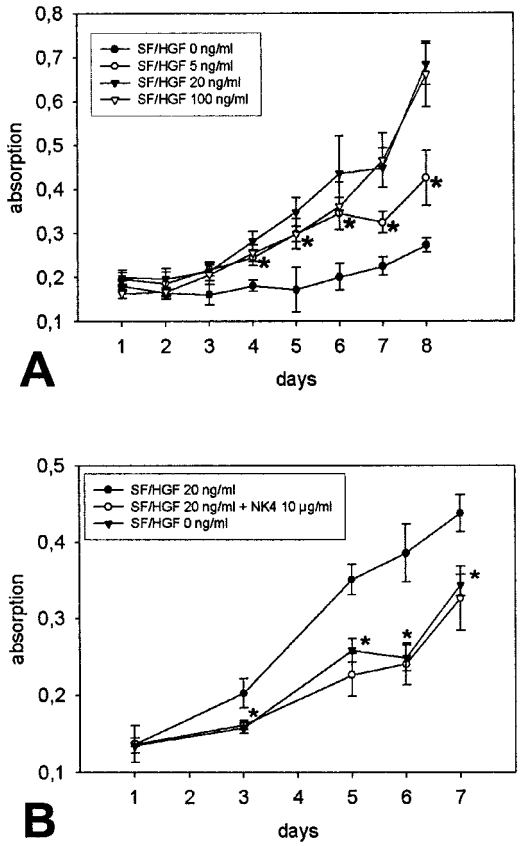

Fig. 5 Inhibition of SF/HGF-induced proliferation of HUVECs by NK4. *A*, cell proliferation was determined over 8 days using a colorimetric assay. ∗ mark the lowest concentration of SF/HGF required to induce significant stimulation. Maximum stimulation of proliferation was observed at 20 ng/ml SF/HGF on most days analyzed. *B*, proliferation was stimulated by 20 ng/ml SF/HGF and was inhibited by coaddition of NK4. Complete inhibition was observed at 10 μg/ml NK4; ∗ indicate significant inhibition.

have lost SF/HGF expression *in vitro* (5, 14). U-87 MG is one of the few glioblastoma cell lines that have retained SF/HGF expression; therefore, we selected it as a model to analyze the effects of NK4 *in vivo*. The results of our study demonstrate that local treatment with NK4 inhibits orthotopic growth of U-87 MG xenografts in nude mice. Tumor volume in NK4-treated animals was reduced by 61.1% when treatment was initiated on day 1 after tumor cell implantation, and an almost identical result was obtained when treatment was targeted against already established tumors, starting treatment on day 7. Treatment was well tolerated, and no signs of toxicity were observed.

We identified several potential mechanisms for the antitumor effect of NK4, including direct effects on tumor cells as well as antiangiogenic effects. The proliferative activity of the tumor cells *in vivo* was significantly reduced compared with controls. Correspondingly, the SF/HGF-induced proliferation of U-87 MG cells *in vitro* was completely inhibited by an ~750-fold molar excess of NK4. These findings suggest that treatment with NK4 can directly inhibit tumor cell proliferation.

Studying glioma cell migration, we observed recently that among 14 different tumor-associated growth factors (including transforming growth factor-α, -β, fibroblast growth factor-1, -2, platelet-derived growth factor, and others), SF/HGF clearly had the strongest motogenic effect on different glioblastoma cell lines.[3] In the present study, NK4 inhibited SF/HGF-induced migration of U-87 MG cells *in vitro*, and we could demonstrate that autocrine stimulation of cell motility was sensitive to inhibition by NK4, suggesting that antagonization of tumor cell migration/invasion may also contribute to the antitumor effect *in vivo*.

In addition to the antimitogenic and antimotogenic effects, we observed a proapoptotic effect of NK4 *in vivo*. In a previous study, SF/HGF was shown to protect glioblastoma cells against apoptosis by different antiapoptotic mechanisms (15). These findings suggest that a proapoptotic component could also be part of the antiglioma activity of NK4 *in vivo*.

SF/HGF not only acts on tumor cells but also on vascular endothelial cells, which express the MET receptor, and it induces angiogenesis *in vivo* (16, 17). Overexpression of SF/HGF in glioma cells was shown to promote tumor vascularization and growth *in vivo* (7). In the present study, we observed a significant reduction of vessel density in tumors from NK4-treated mice, suggesting that inhibition of angiogenesis is also part of its *in vivo* activities. This finding is additionally supported by our *in vitro* observations showing that NK4 inhibited SF/HGF-induced endothelial cell migration and proliferation in a dose-dependent fashion.

Interestingly, recent studies suggest that the antiangiogenic effects of NK4 are not only mediated through antagonization of SF/HGF by competitive binding to the MET receptor. The NK4 molecule has significant structural homology with other kringle-containing proteins, and from some of these proteins, kringle-fragments with antiangiogenic activity have been generated. These include angiostatin as a fragment of plasminogen (18), and the second kringle domain of prothrombin (19), suggesting that a structural motif conserved in some kringle domains could be responsible for the antiangiogenic activity. In line with this hypothesis, NK4 was shown to inhibit vascular endothelial growth factor- and fibroblast growth factor 2-induced endothelial cell migration and proliferation even in the absence of SF/HGF (20). Similar effects were obtained using K1–4, a molecule that lacks the NH_2-terminal hairpin domain, which is essential for MET receptor binding but contains all four of the kringle-domains (21). These findings suggest that MET is only partially involved in the antiangiogenic effects of NK4 and that interference with other receptor systems might additionally contribute to its inhibitory effects on endothelial cells.

NK4 was used previously in different rodent models to treat other types of tumors, including pancreatic, gallbladder, lung, and mammary cancer (20, 22, 23). In these models, NK4 inhibited tumor growth, angiogenesis, and metastatic spread. The present study is the first to demonstrate antitumor activity of NK4 in an orthotopic glioblastoma model. Compared with other agents that interfere with the SF/HGF-MET system, NK4 in the local delivery paradigm currently seems the most prom-

[3] M. A. Brockmann *et al.*, unpublished observations.

ising molecule regarding applicability in glioma patients. Other investigators have used anti-SF/HGF antibodies (24) or hammerhead ribozymes (25, 26) locally and systemically to antagonize the SF/HGF-MET system *in vivo*. However, monoclonal antibodies were only effective in combinations of three different ones, and the application of ribozymes required complex delivery systems such as liposomes or viruses. Another study showed that transfection of NK2, a fragment containing the NH_2-terminal hairpin domain and two kringles of SF/HGF, into glioblastoma cells also caused tumor growth inhibition *in vivo* (27). However, NK2 and also NK1, both of which occur also as natural splice variants of SF/HGF, are only partial antagonists of HGF/SF and display significant agonistic activity. In comparison, NK4 has the advantage of acting strictly antagonistic, which makes it the more promising candidate for glioma treatment.

NK4 is a relatively large molecule; therefore, we injected it distally to the blood-brain barrier. Local therapy of glioblastomas grown in the brain of small rodents used to be difficult. Repeated stereotactic procedures had to be applied to accomplish this task. However, these techniques were time consuming, and the placement of the animals could not always be repeated perfectly, so that some deviation of the injection site was practically unavoidable. In addition, daily reopening of the skin to expose the burr hole for daily stereotactic treatment would most certainly cause severe wound healing problems. To circumvent these problems, Lal *et al.* (12) recently developed the so-called guide-screw system. We introduced several modifications to the system regarding the implantation site and screw fixation to make it more reliable and comfortable to use. In the present study, we showed for the first time that the guide-screw system facilitates daily intratumoral injections over a period as long as 3 weeks without any misinjections or problems such as loosening of the screws.

The concept of local glioma therapy is gaining increasing popularity among clinicians. Glioma progression is usually confined to the brain, and the tumors do not metastasize, which makes them ideal candidates for local intracavitary or interstitial therapy. Intracavitary treatment modalities using chemotherapy and radioimmunotherapy have already proven effective when administered after tumor resection (28, 29). Especially larger molecules that do not efficiently permeate the blood-brain barrier lend themselves to local interstitial application (30). The present study shows that local interstitial treatment with NK4 can inhibit intracerebral glioblastoma growth *in vivo*, most likely by antagonizing tumor cell proliferation, migration, promotion of apoptosis, and inhibition of tumor angiogenesis. Given the strong up-regulation of SF/HGF and MET in malignant human gliomas *in vivo*, NK4 appears to be a promising antiglioma agent that may effectively complement the current conventional strategies.

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