

A Novel One-Armed Anti-c-Met Antibody Inhibits Glioblastoma Growth *In vivo*

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Abstract Purpose: Expression of the receptor tyrosine kinase c-Met and its ligand scatter factor/hepatocyte growth factor (SF/HGF) are strongly increased in glioblastomas, where they promote tumor proliferation, migration, invasion, and angiogenesis. We used a novel one-armed anti-c-Met antibody to inhibit glioblastoma growth *in vivo*.

Experimental Design: U87 glioblastoma cells (c-Met and SF/HGF positive) or G55 glioblastoma cells (c-Met positive and SF/HGF negative) were used to generate intracranial orthotopic xenografts in nude mice. The one-armed 5D5 (OA-5D5) anti-c-Met antibody was infused intratumorally using osmotic minipumps. Following treatment, tumor volumes were measured and tumors were analyzed histologically for extracellular matrix (ECM) components and proteases relevant to tumor invasion. Microarray analyses were done to determine the effect of the antibody on invasion-related genes.

Results: U87 tumor growth, strongly driven by SF/HGF, was inhibited >95% with OA-5D5 treatment. In contrast, G55 tumors, which are not SF/HGF driven, did not respond to OA-5D5, suggesting that the antibody can have efficacy in SF/HGF-activated tumors. In OA-5D5-treated U87 tumors, cell proliferation was reduced >75%, microvessel density was reduced >90%, and apoptosis was increased >60%. Furthermore, OA-5D5 treatment decreased tumor cell density >2-fold, with a consequent increase in ECM deposition and increased immunoreactivity for laminin, fibronectin, and tenascin. Microarray studies showed no increase in these ECM factors, rather down-regulation of urokinase-type plasminogen activator and matrix metalloproteinase 16 in glioblastoma cells treated with OA-5D5.

Conclusions: Local treatment with OA-5D5 can almost completely inhibit intracerebral glioblastoma growth when SF/HGF is driving tumor growth. The mechanisms of tumor inhibition include antiproliferative, antiangiogenic, and proapoptotic effects.

Glioblastomas represent the most common type of brain tumor. Currently available treatment options, such as operation, radiotherapy, and chemotherapy, can only minimally prolong life expectancy, with most patients diagnosed with a glioblastoma usually dying within 1 year. The development of novel treatment strategies is therefore imperative. Recent clinical trials showed that antibodies against a variety of growth factors and their receptors can inhibit the progression of different cancer types. The promise of antibody therapeutics in providing targeted cancer treatment has been validated by

several effective antibodies against growth factors and their receptors alike. For example, directly targeting receptors with oncogenic potential, such as targeting HER-2 with the anti-HER-2 antibody trastuzumab (Herceptin) for treatment of breast cancer, or targeting epidermal growth factor receptor with the anti-epidermal growth factor receptor antibody cetuximab (Erbix) for treatment of metastatic colorectal cancer or non-small cell lung cancer (1). Targeting the ligand of factors critical for cancer progression has also been validated by the use of drugs, such as the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab (Avastin), which has been shown to inhibit the progression of colorectal, breast, and lung cancer (1). To date, no successful antibody therapy has yet been developed for the treatment of malignant gliomas. However, given the therapeutic success of antibodies against other cancer types, this seems to be a potentially promising strategy also for brain tumors.

The multifunctional growth factor scatter factor/hepatocyte growth factor (SF/HGF) and its receptor c-Met are important mediators of brain tumor growth and angiogenesis (2, 3). The SF/HGF molecule is a heterodimer composed of a 69-kDa α -chain containing an NH₂-terminal hairpin domain and four kringle domains, linked by a disulfide bridge to a 34-kDa serine protease-like β -chain. The c-Met receptor tyrosine kinase is

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Received 6/29/05; revised 7/20/06; accepted 8/3/06.

Grant support: Deutsche Forschungsgemeinschaft grant LA 1300/2-1 (K. Lamszus and M. Westphal).

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doi:10.1158/1078-0432.CCR-05-1418

encoded by the *c-met* proto-oncogene and has been widely implicated in tumor progression and invasion (4). Both SF/HGF and c-Met are overexpressed in human glioblastomas, and expression levels correlate with glioma malignancy grade and vascularity (3, 5–8). Overexpression of SF/HGF and/or c-Met promotes glioma growth and angiogenesis *in vivo* (9). *In vitro*, SF/HGF stimulates glioma and endothelial cell migration and proliferation as well as endothelial tube formation (8, 10).

Previous attempts to inhibit the SF/HGF:c-Met axis using antibody-based approaches have been challenging for a variety of reasons. First, targeting SF/HGF with single monoclonal antibodies was found previously to be ineffective. Cao et al. (11) used monoclonal antibodies against SF/HGF to inhibit glioma growth *in vivo*. However, the effects of the individual antibodies were small, and they were only effective when three antibodies were combined, suggesting that single antibodies against SF/HGF could not fully block SF/HGF:c-Met binding. Two recent articles, however, have shown that novel monoclonal antibodies against SF/HGF can have higher potency as single-agent therapeutics (12, 13). Second, targeting c-Met with antibodies has been difficult because most antibodies have intrinsic agonistic activity (14, 15). However, recently, a one-armed (OA) variant of the anti-c-Met antibody 5D5 (16) was developed at Genentech (South San Francisco, CA), which acts as a pure antagonist and can inhibit the growth of cells dependent on SF/HGF:c-Met autocrine and paracrine signaling.³ In the present study, we have successfully used the monovalent OA-5D5 antibody to potentially inhibit glioma growth in an orthotopic *in vivo* model. We have further identified several mechanisms by which this antibody inhibits glioma growth.

Materials and Methods

Antibodies. The one-armed variant of the anti-human c-Met antibody 5D5 (16) was produced as a recombinant protein in *Escherichia coli*. It is composed of murine variable domains for the heavy and light chains with human IgG1 constant domains. The antibody blocks SF/HGF binding to c-Met in a competitive fashion.³

Primary antibodies for Western blot analysis and immunohistochemistry were rabbit polyclonal antibodies against the c-Met β -chain (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated c-Met pY1230, pY1234, and pY1235 (Biosource, Camarillo, CA), von Willebrandt factor (vWF; DAKO, Glostrup, Denmark), cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), and fibronectin (DAKO) or mouse monoclonal antibodies against the Ki-67 antigen (MIB-1; DAKO), laminin (Lab Vision, Fremont, CA), urokinase-type plasminogen activator (uPA; Calbiochem, San Diego, CA), matrix metalloproteinase (MMP) 16 (Lab Vision), tenascin (Sigma, St. Louis, MO), collagen IV (DAKO), and vimentin (DAKO).

Cell culture. The human U-87 MG (U87) glioblastoma cell line was cultured in DMEM (Life Technologies, Paisley, Scotland) supplemented with 10% FCS and 2 mmol/L L-glutamine. Immediately before implantation into nude mice, cells were trypsinized, washed thrice with DMEM without supplements, and adjusted to a concentration of 10^7 per mL in DMEM containing 0.8% methylcellulose.

Western blot analysis. To analyze the effect of the OA-5D5 antibody on c-Met tyrosine phosphorylation, U87 cells were preincubated with the antibody for 1 hour. SF/HGF was added and incubation was

continued for 10 minutes. Cell lysates were prepared by brief sonication in lysis buffer [150 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), 1% SDS, 5 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 20 mmol/L β -glycerophosphate]. Lysates were cleared from cellular debris by centrifugation, and protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Supernatants were precipitated with methanol and chloroform according to Wessel and Flugge (17). Precipitates were resuspended in buffer containing 5% (v/v) β -mercaptoethanol and denatured at 70°C for 5 minutes. Protein (40 μ g) per lane was resolved by SDS-PAGE on 6% gels. Proteins were blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with Roti-Block (Roth, Karlsruhe, Germany) for 1 hour at room temperature and probed overnight at 4°C with polyclonal antibodies against c-Met or phosphorylated c-Met. Binding was detected using secondary anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibodies (Sigma). Immunoreactive bands were visualized using chemiluminescent SuperSignal substrate (Pierce). Coomassie staining was done to verify equal protein loading and proper transfer.

Orthotopic mouse model. Male, 6- to 8-week-old nude mice (NMRI-nu/nu) were used for the intracerebral implantation of U87 cells as described previously (18). Mice were anesthetized by i.p. administration of ketamine (100 mg/kg body weight) and xylazine (5 mg/kg body weight). A burr hole was drilled into the skull 3.5 mm lateral to the bregma. U87 cells ($5 \times 10^5/5 \mu$ L) or G55 cells ($7 \times 10^4/5 \mu$ L) were slowly injected over 5 minutes into the basal ganglia using a 30-gauge needle attached to a 25- μ L Hamilton syringe.

In the first experiment, osmotic minipumps (Alzet mini-osmotic pump, model 2004, Durect Corp., Cupertino, CA) that maintain a constant flow over 28 days were implanted in the same operation immediately following U87 cell engraftment (day 1). The pump reservoir was filled with the OA-5D5 anti-c-Met antibody concentrated 7.1 mg/mL or with vehicle control [10 mmol/L histidine (pH 5.5)]. The reservoir was connected to an intracranial catheter (Alzet brain infusion kit II, Durect). Posterior to the site of tumor cell implantation, a s.c. tunnel was created, and the pump was pushed forward until it came to lay on the back of the mice. The catheter tip was inserted through the burr hole and placed into the center of the tumor. In the second experiment, the minipumps were implanted 7 days after U87 cell engraftment by the same technique. Twenty-eight days after tumor cell implantation, the animals were killed using CO₂. In a third experiment, nude mice that had received G55 glioma cells were treated with the OA-5D5 anti-c-Met antibody starting on day 7 and sacrificed on day 19. OA-5D5-treated and control groups in all experiments comprised 12 animals each. Institutional guidelines for animal welfare and experimental conduct were followed for all experiments conducted during these studies.

Determination of tumor size. Mouse brains were removed from the cranial cavity, fixed in formalin, bisected coronally, and embedded in paraffin. Serial sections (5- μ m thick) were stained with H&E. The maximum cross-sectional area of the glioblastoma xenografts was determined by computer-assisted image analysis using Leica IM50 software (Leica, Hamburg, Germany). Tumor volumes were estimated using the formula: volume = (square root of maximal tumor cross-sectional area)³. All morphometric analyses were done in a blinded fashion without knowledge of the treatment groups.

Immunohistochemical analyses. Paraffin sections were dewaxed using standard histologic procedures. Sections were incubated with primary antibodies overnight at 4°C. Detection of bound antibodies was done using the EnVision System (DAKO) or Vectastain kit (Vector Laboratories, Burlingame, CA) according to the manufacturers' instructions.

Vessel density was determined by counting the number of blood vessels stained with the anti-vWF antibody in 3 high-power fields (hpf), (1 hpf = 0.031 mm²) in the most densely vascularized "hotspot" area. To analyze the proliferative activity of the tumor cells, the percentage of MIB-1-immunoreactive nuclei was determined in 3 hpf in

³ M. Merchant et al., in preparation.

the most actively proliferating tumor area. The fraction of apoptotic tumor cells was determined by counting tumor cells that expressed cleaved caspase-3 in 3 hpf avoiding necrotic areas. All quantitative evaluations of immunohistochemical staining were done in a blinded fashion on the section most adjacent to the slide that contained the maximal tumor cross-sectional area. For some very small tumors, however, more than one section had to be included in the analysis to be able to analyze 3 different hpf.

Cell proliferation assay. U87 glioblastoma cells were seeded into a 96-well plate (2,000 per well) and cultured overnight. On day 1, cells were washed with PBS and the medium was replaced with DMEM, supplemented with 1% FCS, with and without the OA-5D5 antibody. Medium and antibody were renewed on day 4. Sixuplicate wells were fixed at daily intervals using 3% glutaraldehyde. After 7 days, fixed cells were stained with crystal violet, washed with PBS, and solubilized in 10% sodium dodecylsulfate. The absorbance of the lysate was quantified by reading the absorbance at 540 nm in a multiwell plate reader.

Modified Boyden chamber migration assay. The effects of SF/HGF and the OA-5D5 anti-c-Met antibody on the motility of U87 cells were analyzed using a modified Boyden chamber migration assay as described previously (10). Briefly, SF/HGF and the OA-5D5 antibody 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- μ m pore size Nucleopore filter (Neuroprobe) coated with Vitrogen 100 (Cohesion, Palo Alto, CA). Triplicates of U87 cells were seeded into the upper wells at 1.5×10^4 cells/well in 50 μ L serum-free DMEM medium containing 0.1% bovine serum albumin. After incubation at 37°C for 5 hours, 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 hpf using a $\times 40$ objective.

Apoptosis assay. Apoptosis was analyzed using the cell death detection ELISA^{PLUS} kit (Roche, Mannheim, Germany). U87 cells were seeded into a 96-well plate (1×10^4 per well) and cultured overnight. Cells were washed, the medium was replaced with serum-free DMEM, and the cells were cultured for another 24 hours, after which the OA-5D5 anti-c-Met antibody or staurosporin was added. After 3 or 24 hours, the supernatant was aspirated, cells were lysed, and apoptosis as well as necrosis were determined following the manufacturer's instructions.

Microarray analysis. Expression of extracellular matrix (ECM) and adhesion molecules was analyzed using the GEArray Q Series Human Extracellular Matrix & Adhesion Molecules Gene Array (SuperArray, Frederick, MD). Semiconfluent U87 cells were serum starved for 24 hours. Cells were then stimulated with concentrated U87-conditioned medium with a final concentration of 38.5 ng/mL SF/HGF as determined by ELISA (R&D Systems, Minneapolis, MN). After 24 hours of stimulation, the OA-5D5 anti-c-Met antibody (100 μ g/mL) or buffer was added, and incubation was continued for another 24 hours. Total RNA was prepared using the RNeasy kit (Qiagen, Hilden, Germany). Synthesis of biotin-16-dUTP-labeled cDNA probes was done using the GEArray AmpoLabeling-LPR kit that contains a gene-specific primer mix. Array membranes were prehybridized with denatured salmon sperm DNA and incubated with the heat-denatured cDNA probes overnight at 60°C. Membranes were washed twice using $2 \times$ SSC containing 1% SDS and twice using $0.1 \times$ SSC containing 0.5% SDS. Detection was done using the chemiluminescent detection kit (SuperArray) based on alkaline phosphatase-conjugated streptavidin. Chemiluminescent array images were captured using X-ray films at different exposure times and digitalized using a flatbed desktop scanner. Bioinformatic data analysis was done using Adobe Photoshop, Scanalyze, Microsoft Excel, and the GEArray Analyzer tool as recommended by the manufacturers. Signals for ribosomal protein L13a, which were optical within linear exposition range, were selected for normalization. Cutoffs for regulated genes were set at 0.5- to 2-fold, and results were confirmed by repetition of the entire array experiment.

Real-time PCR analysis. RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized using Super-Script II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Gene expression analyses were done using predesigned and prevalidated Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA) and a 7500 Fast Real-time PCR System (Applied Biosystems).

Statistics. Differences between the OA-5D5 antibody-treated and the control-treated groups, as well as differences in the *in vitro* experiments, were analyzed using an unpaired *t* test or Mann-Whitney rank-sum test. *P*s < 0.05 were considered to be statistically significant.

Results

In vitro effects of the OA-5D5 antibody on U87 response to SF/HGF. Like most human glioblastomas, the U87 cell line expresses high levels of SF/HGF and c-Met, thus constituting an autocrine loop activating the c-Met pathway (19). U87 is also capable of responding *in vitro* to exogenous SF/HGF in a paracrine pathway (19). To test the potency of the OA-5D5 anti-c-Met antibody *in vitro*, several assays were done, including assays to evaluate c-Met tyrosine phosphorylation, cell proliferation, migration, and apoptosis.

U87 cells were first tested for their ability to respond to exogenous SF/HGF by inducing phosphorylation of the c-Met receptor and the ability of OA-5D5 to inhibit this induction. U87 cells were serum starved in the presence or absence of OA-5D5 at 50 or 100 μ g/mL for 1 hour. Following this incubation, SF/HGF was added at 20 or 50 ng/mL for 10 minutes, after which the cells were harvested and lysates were prepared for Western blotting to determine total c-Met and phosphorylated c-Met levels. Addition of OA-5D5 eliminated SF/HGF-induced tyrosine phosphorylation of c-Met in this experiment (Fig. 1).

Second, OA-5D5 was tested for its ability to block SF/HGF-induced U87 cell proliferation *in vitro*. U87 cells were plated with or without the OA-5D5 antibody at 50 μ g/mL, and cell proliferation was monitored over 7 days using a colorimetric cell growth assay. OA-5D5 significantly inhibited the proliferation of U87 cells through days 6 and 7 (*P* < 0.005) compared with cells grown in the absence of the antibody (Fig. 2A).

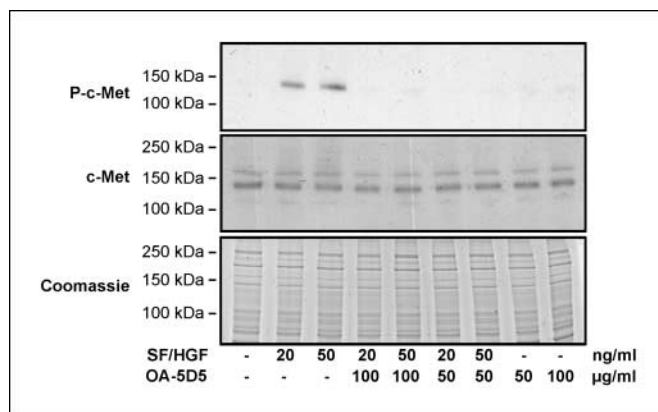


Fig. 1. Inhibition of SF/HGF-induced tyrosine phosphorylation of c-Met by the OA-5D5 antibody. Cells were pretreated with the OA-5D5 antibody for 1 hour and then incubated with SF/HGF for 10 minutes in concentrations as indicated. Western blots were done using an anti-phosphorylated c-Met (*P-c-Met*) antibody and an antibody against the c-Met β -chain. The lower band in the blot probed for c-Met (*middle*) corresponds to the β -chain, whereas the upper band corresponds to the uncleaved pro-form of the receptor.

Third, U87 cell migration was analyzed *in vitro* using a modified Boyden chamber assay. Chemotactic migration of U87 cells was strongly induced by SF/HGF with a maximum response of ~20-fold stimulation observed at 20 ng/mL (Fig. 2B). Stimulation of tumor cell motility by SF/HGF was dose dependently inhibited by coaddition of the OA-5D5 antibody. Inhibition of stimulation by 20 ng/mL SF/HGF was significant at 10 $\mu\text{g/mL}$ OA-5D5 and higher ($P < 0.001$), with stimulation almost completely suppressed by the antibody at 100 $\mu\text{g/mL}$.

Last, the OA-5D5 antibody was evaluated for its ability to induce apoptosis in U87 cells. U87 cells were treated with OA-5D5 at 50 or 100 $\mu\text{g/mL}$ for 24 hours. Staurosporin was used as a positive control with end points at 3 or 24 hours. Cells were harvested, and the fraction of apoptotic tumor cells was determined using a cell death detection ELISA. Following incubation with the antibody at 100 $\mu\text{g/mL}$ for 24 hours, apoptosis was increased 2-fold ($P = 0.001$) over control levels (Fig. 2C). In contrast, the rate of necrosis did not differ between treated cells and controls (data not shown).

In vivo effects of the OA-5D5 antibody on U87 tumor growth. U87 cells were stereotactically engrafted into the caudate/putamen of nude mice on day 1. In the first experiment, osmotic minipumps were implanted immediately after tumor cell engraftment. Pumps were filled with the OA-5D5 antibody or vehicle control, and substances were infused into the center of the tumor via catheters. The experiment was designed to determine the maximal inhibition of tumor growth by treatment with the antibody. In a second experiment, minipumps were implanted on day 7 after tumor cell injection to determine whether treatment was also effective against established tumors.

Animals were killed 4 weeks after injection of U87 cells, when several animals in the control-treated groups had developed weight loss, and brains were analyzed histologically. The mean tumor volume in mice treated with the OA-5D5 antibody was reduced by 96.1% compared with the control group ($0.7 \pm 1.0 \text{ mm}^3$ versus $18.0 \pm 14.5 \text{ mm}^3$; $P < 0.001$; Figs. 3A and B and 4A) when treatment was initiated on day 1. Tumor sizes ranged from 0.0 mm^3 to 3.6 mm^3 in animals treated with the OA-5D5 antibody and from 1.2 mm^3 to 48.9 mm^3 in control-treated mice. In the second experiment, when treatment was initiated on day 7, tumor volume was reduced by 98.7% in OA-5D5-treated mice compared with control-treated mice ($0.4 \pm 0.5 \text{ mm}^3$ versus $28.6 \pm 18.7 \text{ mm}^3$; $P < 0.001$; Figs. 3A and B and 4A). In this experiment, tumor sizes ranged from 0 mm^3 to 1.8 mm^3 in treated animals and from 10.9 mm^3 to 66.8 mm^3 in controls.

To determine whether the OA-5D5 antibody could also be effective against glioblastomas that express high c-Met, but grow independently of SF/HGF, we used the G55 glioblastoma cell line. This cell line expresses c-Met but not SF/HGF mRNA and protein (data not shown), is highly tumorigenic *in vivo*, and is amenable to antiangiogenic treatment (18). Treatment of G55-derived tumors with the OA-5D5 antibody after 7 days of growth *in vivo* had no significant effect on tumor growth (Fig. 4B), suggesting that OA-5D5 acts primarily by blocking SF/HGF binding to and inactivating the c-Met receptor. We further found that incubation with the OA-5D5 antibody *in vitro* had no effect on the proliferation of G55 cells over 9 days (data not shown).

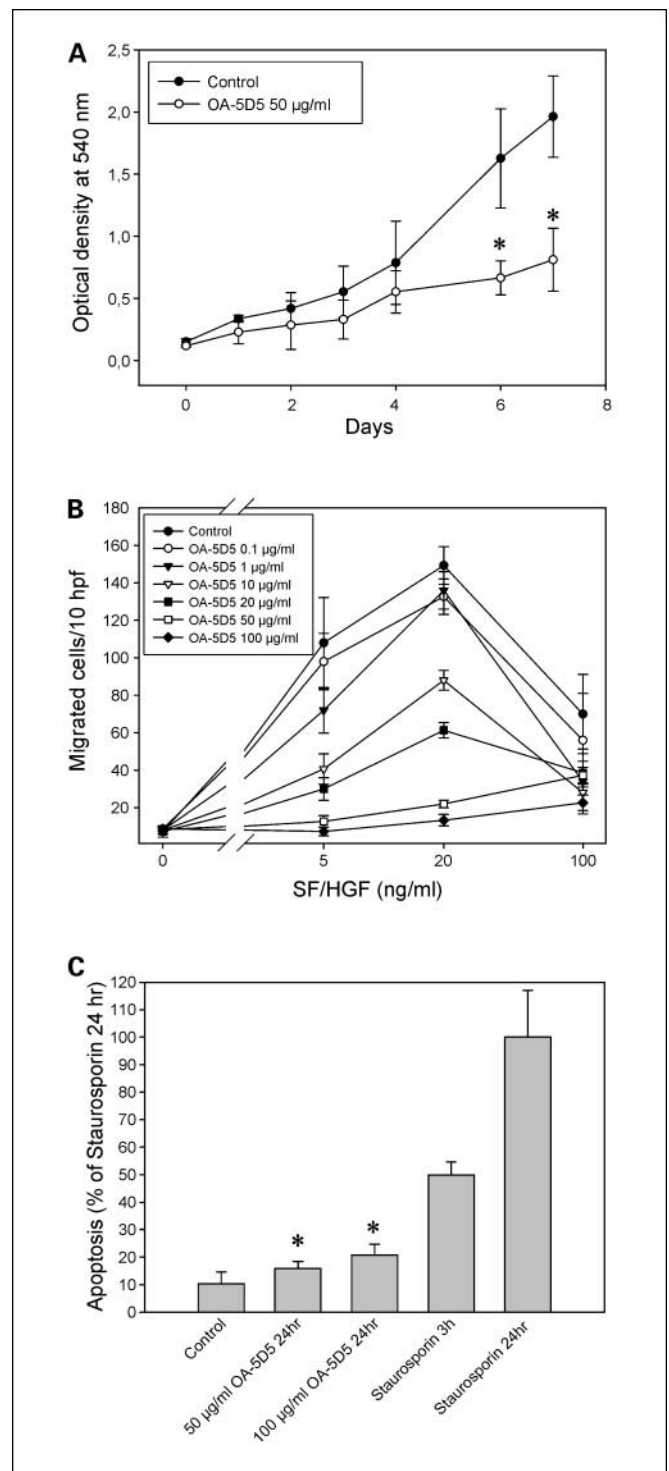


Fig. 2. Effects of the OA-5D5 antibody on U87 cell proliferation, migration, and apoptosis. Cell proliferation was assessed over 7 days using a colorimetric assay. On days 6 and 7, inhibition of proliferation by the OA-5D5 antibody was significant ($P < 0.005$; A). SF/HGF was added to the lower wells of a modified Boyden chamber assay in the presence or absence of the OA-5D5 antibody; U87 cells were seeded into the upper wells; maximum stimulation of chemotactic migration was obtained at 20 ng/mL SF/HGF, and significant inhibition of migration was achieved with the OA-5D5 antibody at concentrations of 10 $\mu\text{g/mL}$ and higher ($P < 0.001$; B). Apoptosis was analyzed using a cell death detection ELISA. After 24 hours of incubation, the percentage of apoptotic cells was increased in the presence of the OA-5D5 antibody at 50 $\mu\text{g/mL}$ ($P < 0.05$) and 100 $\mu\text{g/mL}$ ($P = 0.001$) compared with untreated control cells (C). Points, mean of triplicate determinations; bars, SD (A and B). Columns, mean of sixtriplicate determinations; bars, SD. *, significant effects of the OA-5D5 antibody (A and C); *, left out for clarity (B).

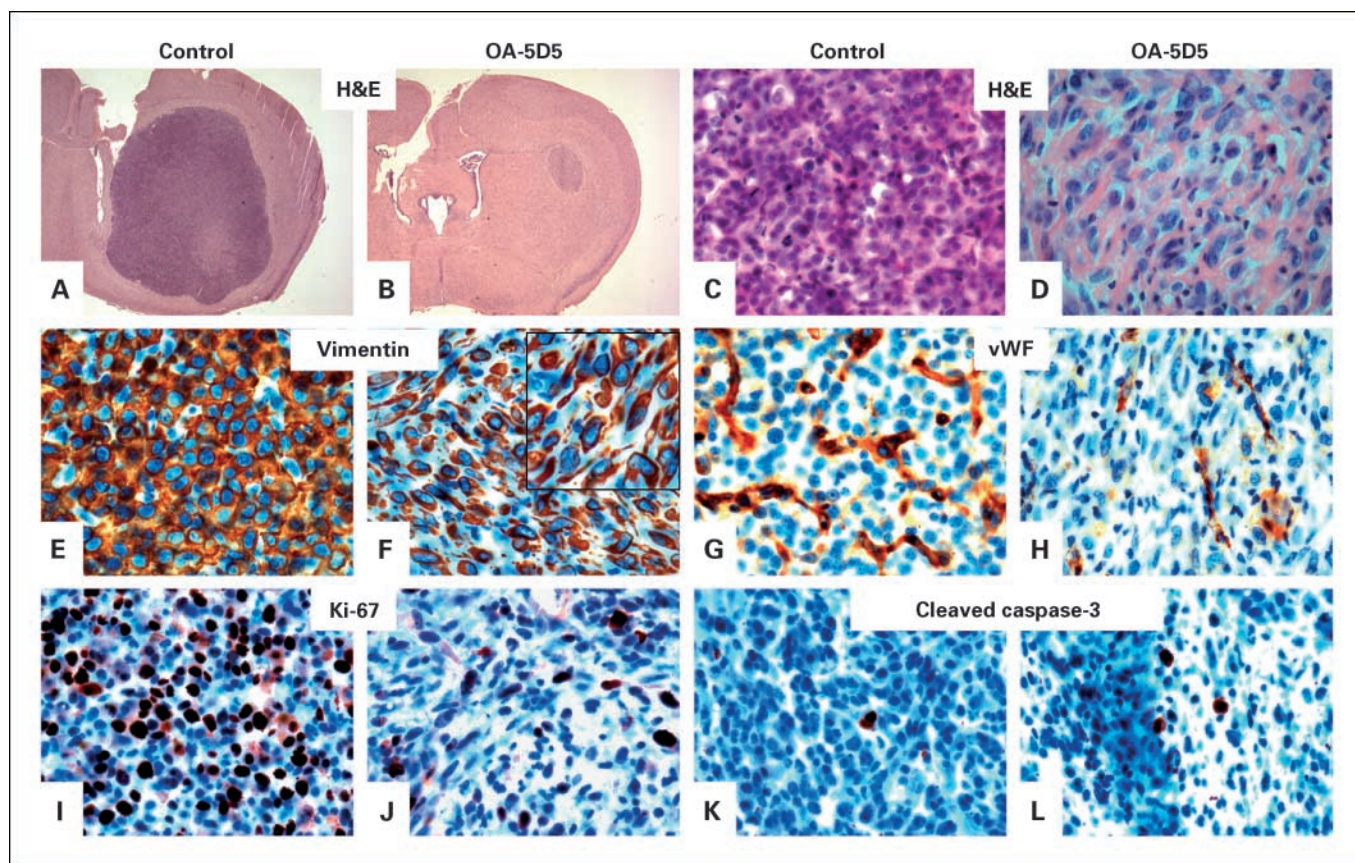


Fig. 3. Histologic examination of U87 tumors in mice treated with the OA-5D5 antibody as of day 7 compared with control-treated tumors. Representative size examples of U87-derived tumors grown intracerebrally in animals that received vehicle (A) or the OA-5D5 antibody (B). Cell density was strikingly higher in control-treated tumors (C) than in OA-5D5 antibody-treated tumors (D). Paraffin sections in (A-D) were stained with H&E. Immunostaining for vimentin showed that intercellular spaces were smaller in control-treated tumors (E) than in OA-5D5 antibody-treated tumors (F); Inset, contains an enlarged view, in which the wide spaces between tumor cells can be discerned more clearly (F). Immunohistochemistry for vWF revealed numerous small capillaries in control-treated tumors (G) but only sparse vasculature in OA-5D5 antibody-treated tumors (H). The percentage of Ki-67-immunoreactive tumor cells was higher in control-treated tumors (I) than in tumors from OA-5D5 antibody-treated animals (J). The fraction of apoptotic tumor cells, as determined by cleaved caspase-3 immunostaining, was lower in control-treated tumors (K) than in OA-5D5 antibody-treated tumors (L). Magnifications, 8-fold (A and B) and 145-fold (C-L).

Characteristic U87 tumor changes following OA-5D5 antibody treatment. To gain insight into the mechanism of action of the OA-5D5 antibody in the orthotopic U87 model, several follow-up histologic and immunohistochemical studies were done. First, a thorough histologic analysis of the OA-5D5- and control-treated tumors was done. When compared with tumors from control-treated mice, OA-5D5 antibody-treated tumors displayed a striking morphologic difference in that the cellularity of these tumors was greatly reduced (Fig. 3C and D). Quantification of nuclei in tumor sections (in 3 hpf) from both groups revealed a 51.8% reduction in cell number in OA-5D5 antibody-treated tumors in the first experiment and a 57.0% reduction in the second experiment ($P < 0.001$; Fig. 4C). Furthermore, tumor cells in OA-5D5 antibody-treated animals were obviously larger than in control-treated tumors with an increase in the amount of eosinophilic material accumulated between tumor cells (Fig. 3C and D). Staining of tumor cells for the intermediate filament vimentin further showed that the extracellular space between individual tumor cells was wider in OA-5D5 antibody-treated tumors versus control-treated tumors, suggesting an increased deposition of ECM in these tumors (Fig. 3E and F).

As SF/HGF and c-Met activity has been linked to VEGF expression (20, 21) and induction of endothelial migration and tube formation (8), intratumoral vessel densities were quantified after staining histologic sections for vWF, which specifically labels endothelial cells. In mice treated immediately with the OA-5D5 antibody, the mean intratumoral microvessel density (in 3 hpf) was reduced by 91.8% relative to tumors in the control group ($P < 0.001$; Figs. 3G and H and 4D). Likewise, initiation of treatment 7 days after xenograft implantation resulted in a 90.6% reduction of the intratumoral microvessel density ($P < 0.001$; Figs. 3G and H and 4D). This is striking as OA-5D5 is not capable of binding murine c-Met, suggesting that inhibition of c-Met activity in the U87 model has an indirect antiangiogenic effect acting through the tumor cells. As expected, the vasculature of the surrounding normal brain was not affected by the OA-5D5 treatment (data not shown).

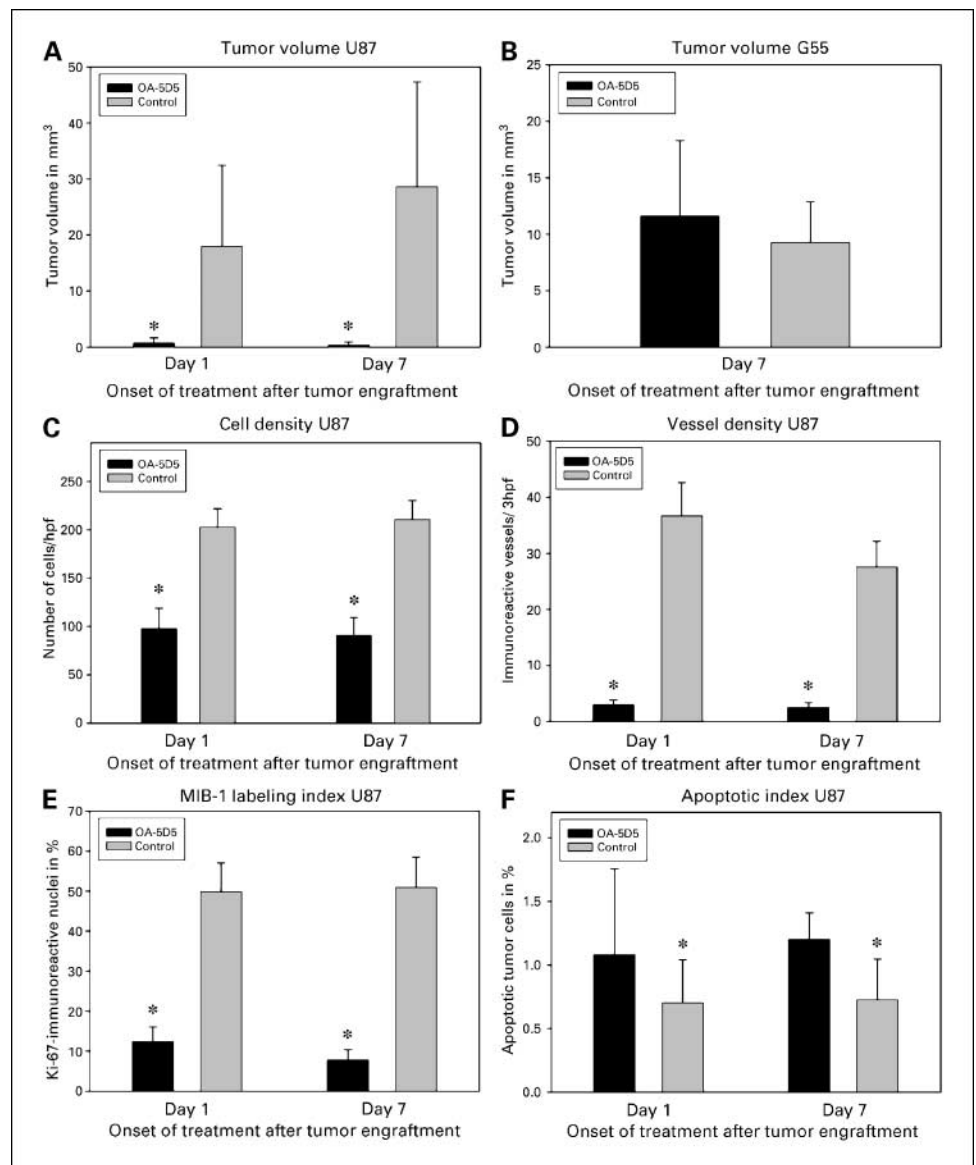
Based on the effects of OA-5D5 on U87 cell proliferation *in vitro*, tumor cell proliferation was analyzed by quantifying the percentage of Ki-67-expressing nuclei (MIB-1 labeling index) on histologic sections. In tumors where OA-5D5 treatment was initiated immediately, the MIB-1 labeling index was reduced by 75.1% ($P < 0.001$) compared with control-treated tumors. When treatment was initiated after tumors were

already established, the proliferative activity was reduced by 84.7% ($P < 0.001$; Figs. 3I and J and 4E), confirming that OA-5D5 has potent antiproliferative effects on U87 cells.

Last, as OA-5D5 treatment moderately increased the number of apoptotic cells *in vitro*, the apoptotic index was quantified in histologic sections as the percentage of cleaved caspase-3-immunoreactive tumor cell nuclei. The overall percentage of apoptotic tumor cells is generally low in the U87 glioblastoma model, and the mean fraction of apoptotic tumor cells did not exceed 2% in OA-5D5-treated as well as in control-treated groups in both experiments (Fig. 4F). Nevertheless, tumors treated with the OA-5D5 antibody displayed a 63.6% higher percentage of apoptotic tumor cells compared with control-treated tumors when treatment was initiated immediately ($P < 0.001$) and a 65.5% higher percentage ($P < 0.001$) when treatment was initiated on day 7 after tumor cell engraftment (Figs. 3K and L and 4F). These results again confirmed the *in vitro* results, suggesting that OA-5D5 is capable of inducing a low to moderate amount of apoptosis in U87 cells.

Expression of ECM molecules and proteases following OA-5D5 treatment. As described above, the morphologic analysis of the tumors on H&E- and vimentin-stained sections showed an obvious widening of the extracellular spaces between tumor cells with an accumulation of eosinophilic material in OA-5D5 antibody-treated mice. We therefore hypothesized that increased amounts of such ECM molecules that are typically secreted by glioma cells might be deposited between tumor cells. Immunohistochemistry for laminin, a major component of the vascular basement membrane, showed that, in control-treated tumors, the vascular network was clearly outlined, whereas laminin was virtually absent on and between tumor cells (Fig. 5A). In contrast, in tumors treated with the OA-5D5 antibody, the tumor cells as well as the ECM were also immunoreactive for laminin, so that vessels could no longer be distinguished from tumor cells (Fig. 5B). Likewise, staining for fibronectin and tenascin was greatly increased in OA-5D5 antibody-treated tumors and localized not only to the tumor cells but especially also to the extracellular space (Fig. 5C-F). In

Fig. 4. Comparison of tumors treated with the OA-5D5 antibody or with vehicle as of day 1 or day 7. Volumes of U87 tumors were reduced by 96.1% in mice treated as of day 1 with the OA-5D5 antibody compared with control-treated animals and likewise by 98.7% in mice treated as of day 7 (A). Volumes of G55 tumors were not significantly different in mice treated with the OA-5D5 antibody or vehicle (B). Cell density was reduced by >50% in OA-5D5 antibody-treated U87 tumors compared with control-treated tumors in both experiments (C). Intratumoral microvessel density was reduced by ~90% in U87 tumors of OA-5D5 antibody-treated animals compared with control-treated animals (D). The proliferative activity of the tumor cells was reduced by 75% to 85% in OA-5D5 antibody-treated U87 tumors compared with control-treated tumors (E). The percentage of apoptotic tumor cells was increased by ~65% in OA-5D5 antibody-treated U87 tumors compared with control-treated tumors (F). *, $P < 0.05$. Columns, mean; bars, SD.



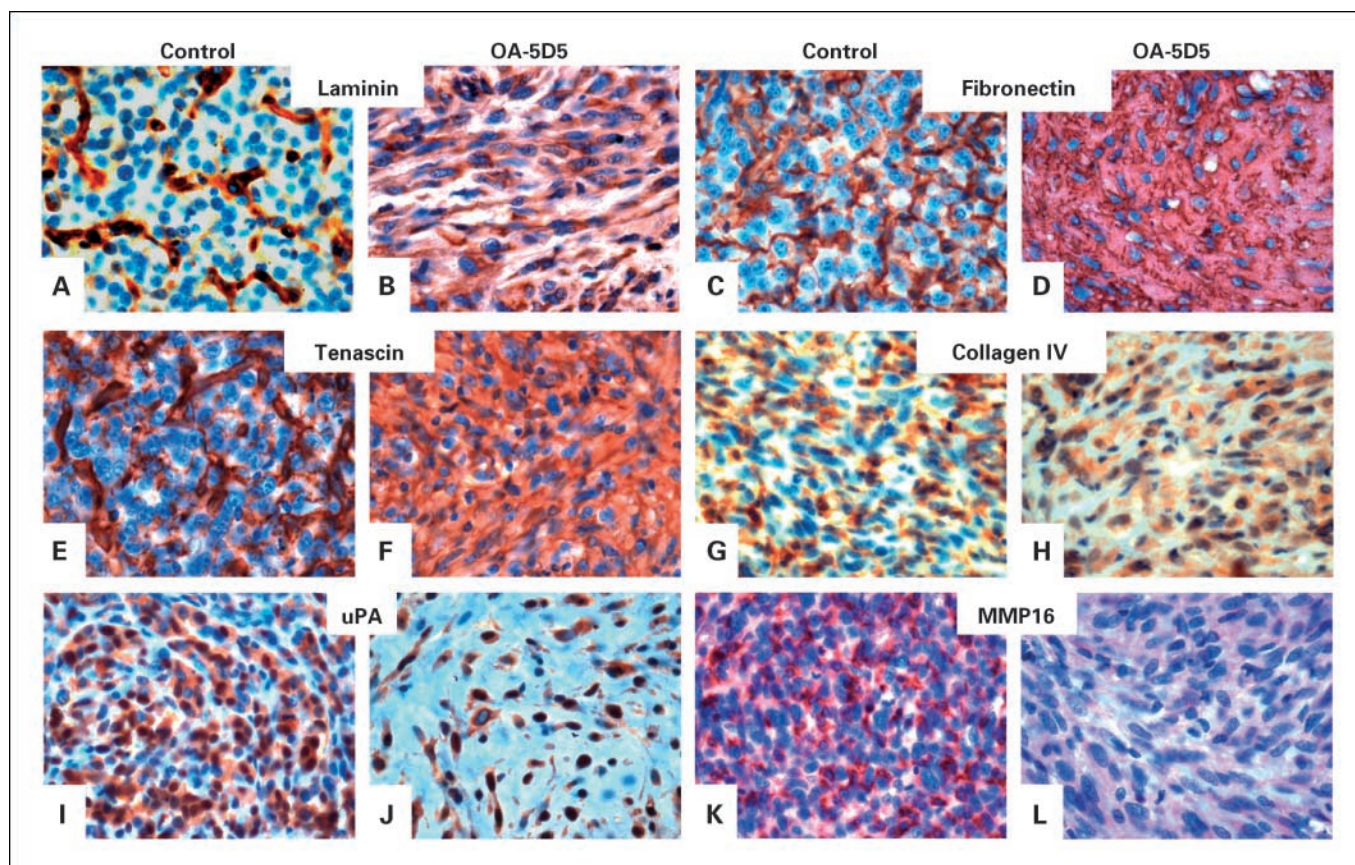


Fig. 5. Immunoreactivity for ECM molecules and proteases in OA-5D5 antibody- and control-treated tumors. Immunoreactivity for laminin (*A* and *B*), fibronectin (*C* and *D*), and tenascin (*E* and *F*) was strikingly increased in OA-5D5 antibody-treated tumors compared with control-treated tumors. Collagen IV immunoreactivity was moderately increased in OA-5D5 antibody-treated tumors compared with control-treated tumors (*G* and *H*). Immunostaining for uPA and MMP16 decreased in OA-5D5 antibody-treated tumors compared with control-treated tumors (*I* and *J*; *K* and *L*, respectively). Magnifications, 145-fold.

control-treated tumors, immunoreactivity for fibronectin and tenascin was almost exclusively associated with blood vessels, whereas collagen IV was mainly associated with the tumor cells. Levels of collagen IV were also increased in OA-5D5 antibody-treated tumors, but staining was less prominent in the extracellular space than that of fibronectin or tenascin (Fig. 5G and H).

Based on these immunohistochemical findings, we hypothesized that antagonization of the SF/HGF:c-Met autocrine loop in U87 glioblastoma cells might have caused an increased expression of ECM molecules by these cells. To test this hypothesis *in vitro*, we treated cultured U87 cells with the OA-5D5 antibody for 24 hours and then did cDNA array analyses. Comparisons of gene expression profiles in the presence and absence of the OA-5D5 antibody, however, showed that neither laminin nor fibronectin or collagen IV were up-regulated by the antibody. Instead, the matrix proteases uPA and MMP16 were down-regulated at least 2- and 5-fold, respectively, in two different array experiments. This observation was confirmed by quantitative real-time PCR analysis using the Taqman system, in which levels of down-regulation were 1.3-fold for uPA ($P = 0.017$) and 1.4-fold for MMP16 ($P < 0.001$). In addition, we analyzed the tumors grown *in vivo* for expression of uPA and MMP16 by immunohistochemistry. A reduction of immunoreactivity for both proteins was found in tumors treated with the OA-5D5

antibody compared with control-treated tumors (Fig. 5I-L). These data show that, although there is not a wholesale increase in several ECM molecules at the transcriptional level, proteins that modify the activity of ECM molecules, such as uPA and MMP16, are down-regulated by OA-5D5 treatment.

Discussion

We have shown that local treatment with the OA-5D5 antibody strongly inhibits glioblastoma growth in an orthotopic model. Treatment was well tolerated, and no signs of toxicity were observed. Previous efforts to develop an antibody against c-Met with antitumor activity were largely unsuccessful because the antibodies tended to have agonistic rather than antagonistic properties (14, 15). This problem has been addressed by cloning of the OA-5D5 antibody consisting of monovalent Fab fused to a human Fc that can be expressed in *E. coli*. In contrast to the bivalent 5D5 antibody, which activates the c-Met pathway and causes receptor internalization and down-regulation on binding (16), OA-5D5 does not agonize c-Met activity but rather acts to potently inhibit SF/HGF binding to c-Met.³

We chose direct intratumoral delivery as route of application for the OA-5D5 antibody because antibodies are large molecules that permeate the blood-brain barrier only insufficiently. The technique of positive pressure intratumoral

infusion is termed convection-enhanced drug delivery. It maximizes compartmental intracerebral drug concentrations while simultaneously overcoming limitations of simple diffusion (22). In addition for gliomas in humans, local delivery is currently considered the most promising route of application for any drug that, due to high molecular weight or poor permeability, needs to bypass the blood-brain barrier. Several promising phase I and II clinical trials were conducted recently using convection-enhanced drug delivery for the delivery of cytostatic drugs or toxin conjugates into gliomas (23). Because the OA-5D5 antibody is a relatively large but stable molecule, convection-enhanced drug delivery was ideally suited to deliver it in our orthotopic model.

Volumes of U87 tumors in mice treated with the OA-5D5 antibody were reduced by up to ~99% (72-fold, when treatment was initiated after 1 week of tumor growth). In contrast, the growth of G55 cells *in vivo* and *in vitro* was not affected by the antibody, suggesting that the antibody has efficacy only in models with active autocrine or paracrine SF/HGF:c-Met signaling. Several previous studies were also able to show inhibition of *in vivo* glioblastoma growth by antagonizing the SF/HGF:c-Met axis. Abounader et al. used hammerhead ribozymes locally and systemically to treat glioblastomas in s.c. and i.c. models (24, 25). In the orthotopic model, tumors were 3-fold smaller in response to direct intratumoral ribozyme delivery and 23-fold smaller in response to i.v. delivery. However, the application of ribozymes required complex delivery systems, such as liposomes or viruses. Our group previously used the SF/HGF antagonist NK4 to treat U87 glioblastomas in an orthotopic model. NK4 is a synthetic molecule comprising the NH₂-terminal hairpin domain and subsequent four kringle domains of SF/HGF that competes with SF/HGF binding of the c-Met receptor and downstream effects on tumor and endothelial cells (26, 27). We achieved 61% inhibition of tumor growth by injecting 25 µg NK4 every day over 2 to 3 weeks through a guide screw into U87 tumors in the brains of nude mice (19). Another study used transfection of NK2, an even smaller fragment of the SF/HGF molecule to inhibit glioblastoma growth *in vivo* (28). However, NK2 is only a partial antagonist of SF/HGF, displaying also significant agonistic activity. Cao et al. (11) used monoclonal anti-SF/HGF antibodies to treat s.c. glioblastoma xenografts; however, the antibodies were only effective in triple combinations. However, two groups have shown recently that single monoclonal antibodies against SF/HGF are capable of inhibiting its ability to bind to c-Met, both showing efficacy against U87 when grown as either a s.c. or an intracranial orthotopic model (12, 13). As shown here with the U87 glioblastoma model, the OA-5D5 antibody is a promising agent with regard to effectiveness and applicability with the unique ability to target the c-Met receptor, disrupting the ability of SF/HGF to bind to the receptor.

We identified several potential mechanisms for the anti-tumor effect of the OA-5D5 antibody, including direct effects on tumor cells as well as effects on the tumor vasculature. Autocrine stimulation of U87 glioblastoma cell proliferation was strongly inhibited by the OA-5D5 antibody *in vitro*, and the proliferative activity of the tumor cells was reduced by about 75% to 85% *in vivo*. Although the effect was modest, the fraction of apoptotic tumor cells was increased by ~65% in tumors treated with the OA-5D5 antibody, and incubation of

U87 cells with the antibody *in vitro* caused increased cell apoptosis. This finding is consistent with known antiapoptotic effects of SF/HGF on glioma cells and other tumor cells *in vitro* (25, 29, 30). We previously found SF/HGF to be the strongest motogen for glioma cells compared with a variety of other different growth factors (31). In keeping with this finding, the OA-5D5 antibody was found to strongly inhibit SF/HGF-induced U87 cell migration in a dose-dependent fashion *in vitro*. Taken together, these findings suggest that OA-5D5 has antiproliferative and proapoptotic effects that contribute to the inhibition of tumor growth *in vivo* and that antimigratory effects could potentially also be of relevance to glioma progression.

We observed a striking alteration of tumor morphology on treatment with the OA-5D5 antibody. Tumor cellularity was reduced by >50%, concurrent with a relative increase in size of the extracellular space. Immunohistochemistry showed a strongly increased deposition of various ECM molecules in OA-5D5-treated tumors, including laminin, tenascin, and fibronectin. Staining of tumor cells for collagen IV was also moderately increased. Unexpectedly, however, we were unable to detect increased expression of these ECM molecules following incubation of U87 cells with the OA-5D5 antibody *in vitro*. Instead, mRNA expression of the secreted protease uPA and of the membrane-type protease MMP16 was down-regulated. Among the substrates cleaved by the uPA-plasmin system or by MMP16 are fibronectin, laminin, and tenascin-C. Therefore, our observations suggest that decreased intratumoral proteolytic activity rather than increased expression is likely to be responsible for the enhanced accumulation of ECM molecules in tumors treated with the OA-5D5 antibody.

We confirmed down-regulation of uPA and MMP16 in antibody-treated tumors by immunohistochemistry. The decrease in uPA levels by blockade of SF/HGF signaling through c-Met is in line with previous reports that showed up-regulation of uPA by SF/HGF in glioma cells. Moriyama et al. (32) described that incubation of glioma cells with SF/HGF *in vitro* resulted in increased expression of uPA. In a study by Lattera et al. (9), overexpression of SF/HGF in rat glioma cells caused increased tumor growth *in vivo*, and SF/HGF-producing gliomas contained 3.5-fold higher levels of uPA than control tumors. Our current findings suggest that treatment with the OA-5D5 antibody can inhibit autocrine up-regulation of uPA by the SF/HGF signaling through c-Met system in U87 cells. Interestingly, uPA can also activate single-chain pro-SF/HGF by proteolytic cleavage to form active two-chain SF/HGF (33). It may therefore be speculated that the depletion of uPA in antibody-treated tumors may cause a relative reduction of active SF/HGF, which would further enhance the antagonistic effects of OA-5D5 or other anti-c-Met therapies.

SF/HGF, c-Met, and uPA were shown to correlate with increasing malignancy grade in human gliomas (3, 6–8, 34, 35). uPA can mediate glioma cell invasion, mainly by activating plasminogen to form plasmin, which in turn activates several pro-MMPs that mediate degradation of the ECM (35). Matrix degradation is considered an essential requirement for glioma cell invasion. Unfortunately, the commonly used models that are based on the engraftment of human glioblastoma cell lines only partially model the growth pattern of gliomas in humans. Xenografted cell lines, including the U87, usually form solid tumors in murine brain rather than diffusely infiltrating

ones. Therefore, it can only be speculated that such a striking increase in ECM deposition, as was observed in our model on treatment with the OA-5D5 antibody, might possibly impair invasion also in truly infiltrative tumors.

Another mechanism that seems to have contributed to the antitumor effect of the OA-5D5 antibody *in vivo* is inhibition of tumor angiogenesis. The intratumoral microvessel density was reduced by >90% by the antibody treatment. Signaling through c-Met is known to directly mediate proangiogenic effects, such as endothelial cell migration, proliferation, and formation of three-dimensional tubes (36). As the OA-5D5 antibody does not recognize murine c-Met, the effects observed in the U87 orthotopic tumor model are likely occurring through modulation of the ability of the tumor to induce angiogenesis. Down-regulation of the uPA proteolysis and MMP proteolysis network may contribute to the decrease in tumor angiogenesis, as proteolysis is a requirement for the sprouting of new capillaries.

To conclude, we have shown here that local treatment with the OA-5D5 anti-c-Met antibody strongly inhibits the growth of glioblastoma cells in an orthotopic model. Mechanisms responsible for this antitumor effect include inhibition of tumor cell proliferation, migration, and angiogenesis as well as down-regulation of proteolytic enzymes and proapoptotic effects. Given the strong up-regulation of SF/HGF and c-Met in malignant human gliomas *in vivo*, the OA-5D5 antibody seems to be a promising anti-glioma agent that may be able to effectively complement the currently available conventional treatment strategies.

Acknowledgments

We thank Roswitha Reusch for competent help with the animal experiments and Sker Freist for expert assistance with the photographic work.

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Clin Cancer Res 2006;12:6144-6152.

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