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Inhibition of intracerebral glioblastoma growth by targeting the insulin-like growth factor 1 receptor involves different context-dependent mechanisms

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Background. Signaling by insulin-like growth factor 1 receptor (IGF-1R) can contribute to the formation and progression of many diverse tumor types, including glioblastoma. We investigated the effect of the IGF-1R blocking antibody IMC-A12 on glioblastoma growth in different in vivo models.

Methods. U87 cells were chosen to establish rapidly growing, angiogenesis-dependent tumors in the brains of nude mice, and the GS-12 cell line was used to generate highly invasive tumors. IMC-A12 was administered using convection-enhanced local delivery. Tumor parameters were quantified histologically, and the functional relevance of IGF-1R activation was analyzed in vitro.

Results. IMC-A12 treatment inhibited the growth of U87 and GS-12 tumors by 75% and 50%, respectively. In GS-12 tumors, the invasive tumor extension and proliferation rate were significantly reduced by IMC-A12 treatment, while apoptosis was increased. In IMC-A12–treated U87 tumors, intratumoral vascularization was markedly decreased, and tumor cell proliferation was moderately reduced. Flow cytometry showed that <2% of U87 cells but >85% of GS-12 cells expressed IGF-1R. Activation of IGF-1R by IGF-1 and IGF-2 in GS-12 cells was blocked by IMC-A12. Both ligands stimulated GS-12 cell proliferation, and IGF-2 also stimulated migration. IMC-A12 inhibited these stimulatory effects and increased apoptosis. In U87 cells, stimulation with either ligand had no functional effect.

Conclusions. IGF-1R blockade can inhibit glioblastoma growth by different mechanisms, including direct effects on the tumor cells as well as indirect anti-angiogenic effects. Hence, blocking IGF-1R may be useful to target both the highly proliferative, angiogenesis-dependent glioblastoma core component as well as the infiltrative periphery.

Keywords: angiogenesis, convection-enhanced delivery, glioma, IGF-1R, invasion.

Signaling by type 1 insulin-like growth factor receptor (IGF-1R) plays a role in the formation, maintenance, and progression of many diverse tumor types.[1] In the central nervous system, IGF-1R has been implicated in fetal and postnatal brain development as well as in brain tumor growth.[2] Overexpression of IGF-1R and its ligands IGF-1 and IGF-2 was detected in human astrocytomas, and activated IGF-1R signaling indicates that this pathway contributes to tumor progression.[3–7] Furthermore, resistance to treatment with epidermal growth factor receptor tyrosine kinase inhibitors in glioblastoma cells can be mediated by IGF-1R activation.[8–10]

IGF-1R is a transmembrane receptor tyrosine kinase that activates the signaling pathways of phosphatidylinositol-3 kinase/Akt and Ras-Raf–mitogen-activated protein kinase.[2] It belongs to the insulin receptor (IR) family, which includes IR, IGF-1R, the hybrid receptor IGF-1R/IR, and IGF-2R. IGF-1R is primarily activated by IGF-1 and IGF-2, but supraphysiological levels of insulin can also stimulate the receptor.[2] Blockade of IGF-1R signaling can induce multimodal antitumor effects, such as pro-apoptotic, antiproliferative, anti-invasive and anti-angiogenic activity. Different types of IGF-1R inhibitors have been developed, including antibodies

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and low-molecular-weight tyrosine kinase inhibitors.[11] Phase II clinical trials with monoclonal anti–IGF-1R antibodies have demonstrated sustained responses in a subset of patients with Ewing sarcoma and thymoma; however, phase III trials with colorectal or lung cancer patients failed to show clinical benefit.[11] In pilot studies of patients with malignant gliomas, IGF-1R antisense oligonucleotide strategies were tested, but responses were only moderate,[12] so that this strategy has not yet been developed beyond phase I. In an ongoing phase I/II trial on patients with recurrent malignant astrocytoma, the efficacy of picropodophyllin, a cyclolignan that inhibits IGF-1R activation, is being evaluated (http://www.clinicaltrials.gov/ct2/show/NCT01721577). Picropodophyllin has demonstrated antitumor activity in several glioma models[13]; however, it possesses also nonspecific activities.

IMC-A12 (cixutumumab) is a fully human monoclonal antibody directed against IGF-1R. It binds IGF-1R with high affinity, blocks its interaction with IGF-1 and IGF-2, and induces receptor internalization and degradation.[14,15] IMC-A12 also binds hybrid IGF-1R/IR receptors on tumor cells but not IRs. IMC-A12 has demonstrated growth inhibition in vivo of several tumor types, such as breast, renal, and pancreatic cancer, and has been tested in phase II clinical cancer trials on patients with sarcomas and adrenocortical carcinomas.[16,17]

In the present study, we demonstrate that the IMC-A12 antibody potently inhibits glioblastoma progression in 2 different orthotopic in vivo xenograft models. Interestingly, we identified different mechanisms of tumor growth inhibition. In a glioblastoma stemlike (GS) cell line–derived xenograft model, which captures the infiltrative aspect of the disease, growth inhibition was achieved by direct inhibition of tumor cell proliferation and invasion. In contrast, in a non-invasive, highly angiogenesis-dependent model, growth inhibition was apparently achieved by a striking reduction of tumor vascularization.

Materials and Methods

Cell Culture

Glioblastoma cell lines U87, U251, T98G, G44,[18] and G55,[18] as well as MCF7 and HepG2 cells, were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) with 10% fetal calf serum (FCS) and 2 mM L-glutamine. Glioblastoma stemlike cell lines GS-8, GS-11, GS-12, and GS-13 were cultured as neurospheres as described previously.[19]

Orthotopic In vivo Tumor Models

Animal experiments were approved by the local authority in Hamburg. U87 xenografts and treatment were performed as described previously.[20] Briefly, 3×10^5 cells in serum-free DMEM were injected stereotactically into the striatum of 6- to 8-week-old anesthetized Naval Medical Research Institute /Foxn1nu mice. After 7 days, Alzet osmotic minipumps (model 2004, flow rate 0.25 μL/h; Durect) were implanted subcutaneously.[20] Pump reservoirs were filled with IMC-A12 (10 mg/mL, provided by Imclone Systems) or saline as vehicle control ($N = 11$ per group). Reservoirs were connected to an intracranial catheter (Alzet Brain Infusion Kit II) placed in the center of the tumor. Treatment was continued over 3 weeks, after which mice were killed using CO_2. GS-12 xenografts were generated by implanting 1.5×10^5 dissociated cells into the striatum ($N = 12$ per group). IMC-A12 treatment was initiated 8 weeks later using osmotic minipumps and continued over 4 weeks.

Determination of Tumor Size

Serial sections from formalin-fixed, paraffin-embedded brains were stained with hematoxylin and eosin. U87 tumor sizes were determined using digital image analysis, and volumes were calculated as described[20] using the formula: volume = (square root of maximal tumor cross-sectional area)^3. Tumor burden of GS-12 xenografts was determined by a technique for quantifying tumor burden of diffusely infiltrating tumors described previously[21] and in the Supplementary Materials and Methods section.

Immunohistochemical Analysis

Paraffin section from xenograft tumors, human glioblastomas, and a tissue microarray (TMA) were immunostained with antibodies against CD34, cleaved caspase-3, Ki-67, and IGF-1R as described previously[21,22] and as detailed in the Supplementary Materials and Methods section.

Quantitative PCR Analysis

RNA extraction, cDNA transcription, and real-time PCR analyses were performed as described previously[19] using the following TaqMan gene expression assays: IGF-1R: Hs99999020_mL; IGF-1: Hs01547656_mL; IGF-2: Hs04188276_mL; vascular endothelial growth factor (VEGF) α: Hs00900055_mL; RPL13A: Hs01578912_mL (Applied Biosystems). Relative amounts of target mRNA were normalized to RPL13A, values were calibrated according to the delta delta cycle threshold method, and relative quantity values were calculated.

Flow Cytometry

To detect IGF-1R on glioblastoma cell lines, flow cytometry was performed using IMC-A12 as primary antibody. Experimental details are provided in the Supplementary Materials and Methods section.

Western Blot Analysis

GS-12 cells were cultured in neural basal medium with insulin-free B27 and 4 nM insulin (Sigma-Aldrich) (low insulin medium). Cells were incubated with or without IGF-1, IGF-2, and IMC-A12 prior to analysis. IGF-1R, phospho–IGF-1R, IGF-1, IGF-2, and α-tubulin were analyzed by reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting as detailed in the Supplementary Materials and Methods section.

Cell Proliferation Assay

Cells were seeded at 2500 cells/well into 96-well plates. U87 cells were serum starved overnight and subsequently supplemented with 1% FCS. GS-12 cells were transferred to low insulin

medium at least 1 week prior to the experiment. IGF-1, IGF-2, or IMC-A12 was added, and incubations were continued over 4 days for U87 cells and over 8 days for GS-12 cells. Fresh medium containing growth factors was added after 4 days to GS-12 cells. Proliferation was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega).

Modified Boyden Chamber Migration Assay

Glioma cell migration was analyzed using modified Boyden chamber assays as described.[19] Briefly, neural basal medium / 0.1% bovine serum albumin with IGF-1 or IGF-2 was added to the lower wells of a 96-well modified Boyden chamber (Neuro Probe). For inhibition experiments, cells were pre-incubated with IMC-A12 for 48 h, and IMC-A12 was added to the lower and upper wells. Wells were covered with a laminin-coated 8-μm pore size filter. Quintuplicates of 1.5×10^4 cells were seeded into the upper wells. Migrated GS-12 and U87 cells were stained and counted after 20 h and 6 h, respectively, of incubation.

Apoptosis Assay

Apoptotic cell death in response to IMC-A12 or etoposide (Merck) was quantified using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen) as described in the Supplementary Materials and Methods section.

Statistical Analyses

Statistical comparisons among different treatments in vivo and in vitro were performed using the unpaired *t*-test for normally distributed samples or a Mann–Whitney rank-sum test for not normally distributed samples and the SigmaStat program.

Results

In vivo Effects of IMC-A12 on Glioblastoma Growth

The efficacy of the IMC-A12 antibody was evaluated in 2 different orthotopic models. In the first model, we used the U87 cell line, which gives rise to well-delineated, nodular, non-invasive, fast-proliferating tumors in the brains of nude mice and was reported to express IGF-1R.[13,23–26] U87 tumors typically contain necroses, and their continuous growth strongly depends on angiogenesis, hence this model mimics the solid core component of human glioblastomas. Seven days after stereotactic cell engraftment, osmotic minipumps were implanted, delivering IMC-A12 or vehicle control intratumorally. Animals were killed after 3 weeks of treatment. At this point, 7 of the 11 animals in the control group but none in the IMC-A12 treatment group had developed progressive weight loss (≥10%), and 3 of the 7 mice had additional motor impairment. Histological analyses showed that the mean tumor volume in mice treated with IMC-A12 was reduced by 73.7% compared with the control group (3.3 ± 3.4 mm^3 vs 12.6 ± 7.7 mm^3, $P < .01$; Fig. 1A).

In the second model, we used the GS-12 cell line, which grows slowly but highly invasively and typically infiltrates the contralateral hemisphere through the corpus callosum and anterior commissure, but rarely contains necroses, thus reflecting the invasive component of human glioblastomas. Eight weeks after cell engraftment, minipumps were implanted, and treatment was carried out over 4 weeks, after which the animals were killed. By that time, 4 of the 12 animals in the control group but none in the IMC-A12–treated group had developed weight loss. Because of the extensive diffuse invasion of the tumor cells, we used a previously developed technique to quantify tumor burden also in regions where tumor cells and normal cells are intricately admixed.[19,21] We quantified the net area taken up by tumor cell nuclei in 36 different landmark areas at 6 defined coronal levels, each with 6 defined regions of analysis. This analysis revealed that IMC-A12 treatment caused significant inhibition of tumor infiltration in all 36 regions (Fig. 1B). Interestingly, tumor cell densities were reduced to a significantly greater extent in remote areas than in central regions. For example, at the most distant sites (level 6, regions 5 and 6) reductions were significantly greater than at the injection site (level 2, region 4) ($P = .01$ for each comparison). When the results for all 36 regions were pooled, the cumulative tumor burden in IMC-A12–treated animals was reduced by 50.3% compared with the control group ($P < .001$; Fig. 1B).

Mechanisms of Tumor Growth Inhibition In vivo

To gain insight into the mechanisms of action of the IMC-A12 antibody in the 2 different models, immunohistochemical studies were performed. Tumor cell proliferation was quantified as percentage of Ki-67–expressing nuclei. IMC-A12 treatment had a moderate inhibitory effect on the proliferation rate of U87 cells in vivo, which was significant when analyzed in 4 randomly selected high power fields ($21.4\% \pm 4.1$ vs 26.2 ± 4.8, $P < .05$; Fig. 1A). In GS-12 tumors, proliferation analyzed in central hot spot areas was markedly reduced in IMC-A12–treated tumors compared with controls ($25.7 \pm 7.9\%$ vs $35.3 \pm 9.5\%$, $P < .05$; Fig. 1B).

Apoptosis was quantified by determining the percentage of cleaved caspase-3–immunoreactive tumor cell nuclei. The proportion of apoptotic cells was generally low and did not exceed 1% in both models. IMC-A12 had no significant effect on apoptosis in U87 tumors, whereas the proportion of apoptotic cells in IMC-A12–treated GS-12 tumors was elevated 2.6-fold versus controls ($P < .05$; Fig. 1A and B).

As IGF-1R is also expressed on endothelial cells and its activation has been linked to angiogenesis, we compared intratumoral microvessel densities by counting CD34-immunoreactive blood vessels. Interestingly, microvessel density was reduced by 37.9% in U87 tumors treated with IMC-A12 compared with controls ($P < .05$; Fig. 1A). In contrast, IMC-A12 had no effect on the vascularization of GS-12 xenografts, and these highly invasively growing tumors had progressed largely independently of angiogenesis (Fig. 1B). No dividing endothelial cells or microvascular proliferations could be detected, and tumors generally displayed a reduced microvessel density compared with normal murine brain due to the expansion of neoplastic cells between the preexistent host vasculature, consistent with previous studies.[21,27]

We further assessed the IGF-1R expression status in xenograft tumors by immunohistochemistry. Only a few scattered tumor cells in U87 tumors displayed IGF-1R immunoreactivity, whereas the majority of GS-12 cells exhibited strong staining

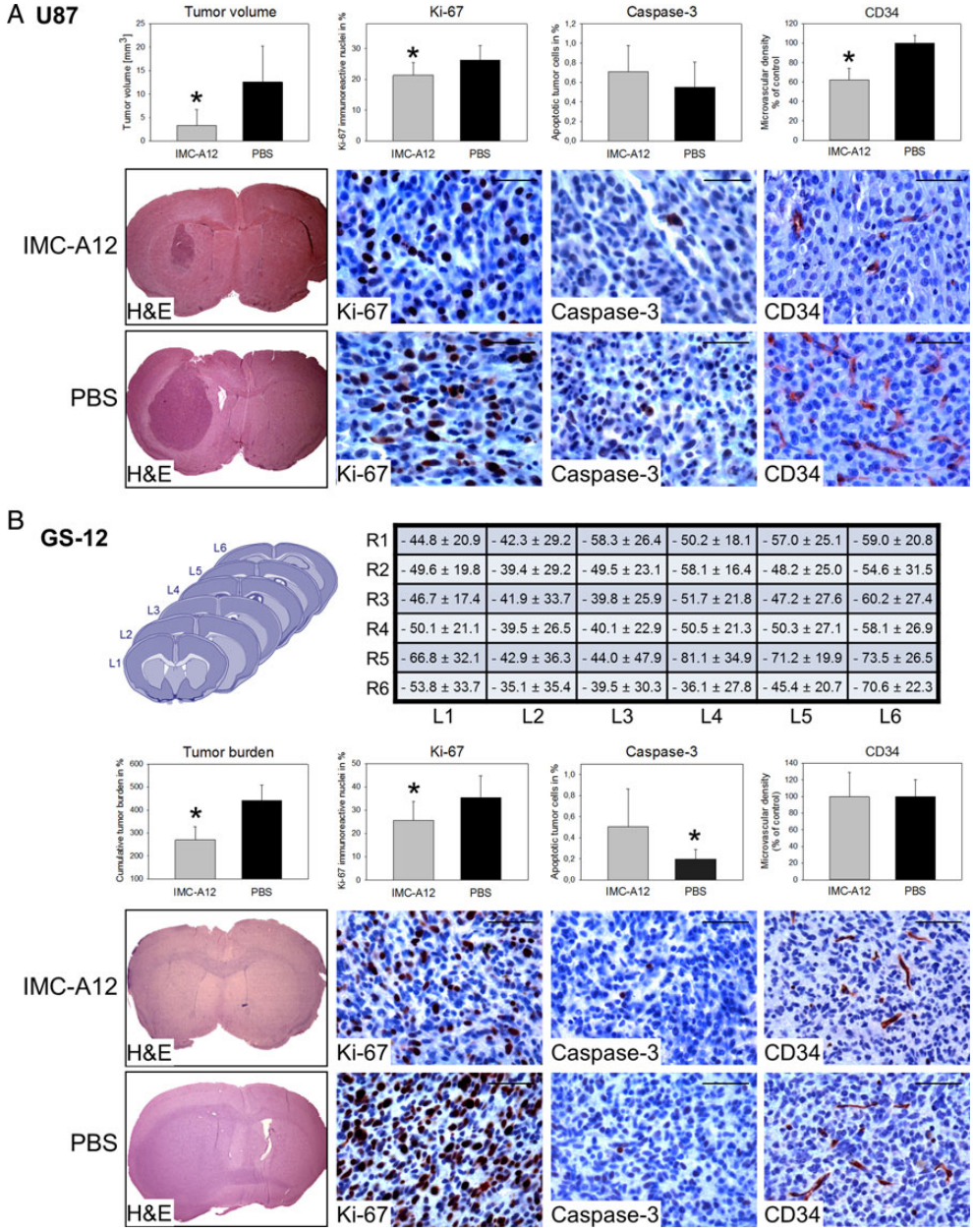

Fig. 1. Effect of IMC-A12 on glioblastoma growth in vivo. (A) Mean tumor volume, microvessel density (CD34), and proliferation (Ki-67) were reduced in IMC-A12–treated U87 tumors. (B) Tumor burden in mice engrafted with GS-12 cells was assessed by analyzing 36 landmark areas (at 6 coronal levels, L1–L6), evaluated in 6 defined regions (R) each. Values in the table represent mean percent reduction ± SD of tumor cell burden in 36 areas in IMC-A12–treated animals versus controls. Growth inhibition was significant in all regions ($P < .05$) and was more pronounced distant from the injection site (L2, central striatum). Tumor cell proliferation was reduced in IMC-A12–treated mice, while apoptosis (caspase-3) was increased. Values are means ± SD; asterisks indicate significance ($P < .05$); size bars are 50 μm.

(Fig. 2A). Conversely, vascular immunoreactivity was more prominent in U87 than in GS-12 tumors. In particular, dilated vessels in U87 tumors displayed strong reactivity, while staining was weak or absent on delicate capillaries in GS-12 tumors.

These results suggest that inhibition of U87 xenograft growth by IMC-A12 is mainly achieved by inhibition of tumor angiogenesis. In contrast, IMC-A12 appears to have a direct effect on GS-12 cells, causing inhibition of tumor cell proliferation and invasion as well as increased apoptosis, while angiogenesis is largely irrelevant in this highly invasive model.

Insulin-like Growth Factor 1 Receptor and Ligand Expression Analyses

To further elucidate the mechanisms that underlie the differential effects of IMC-A12 on GS-12 versus U87 cells, we

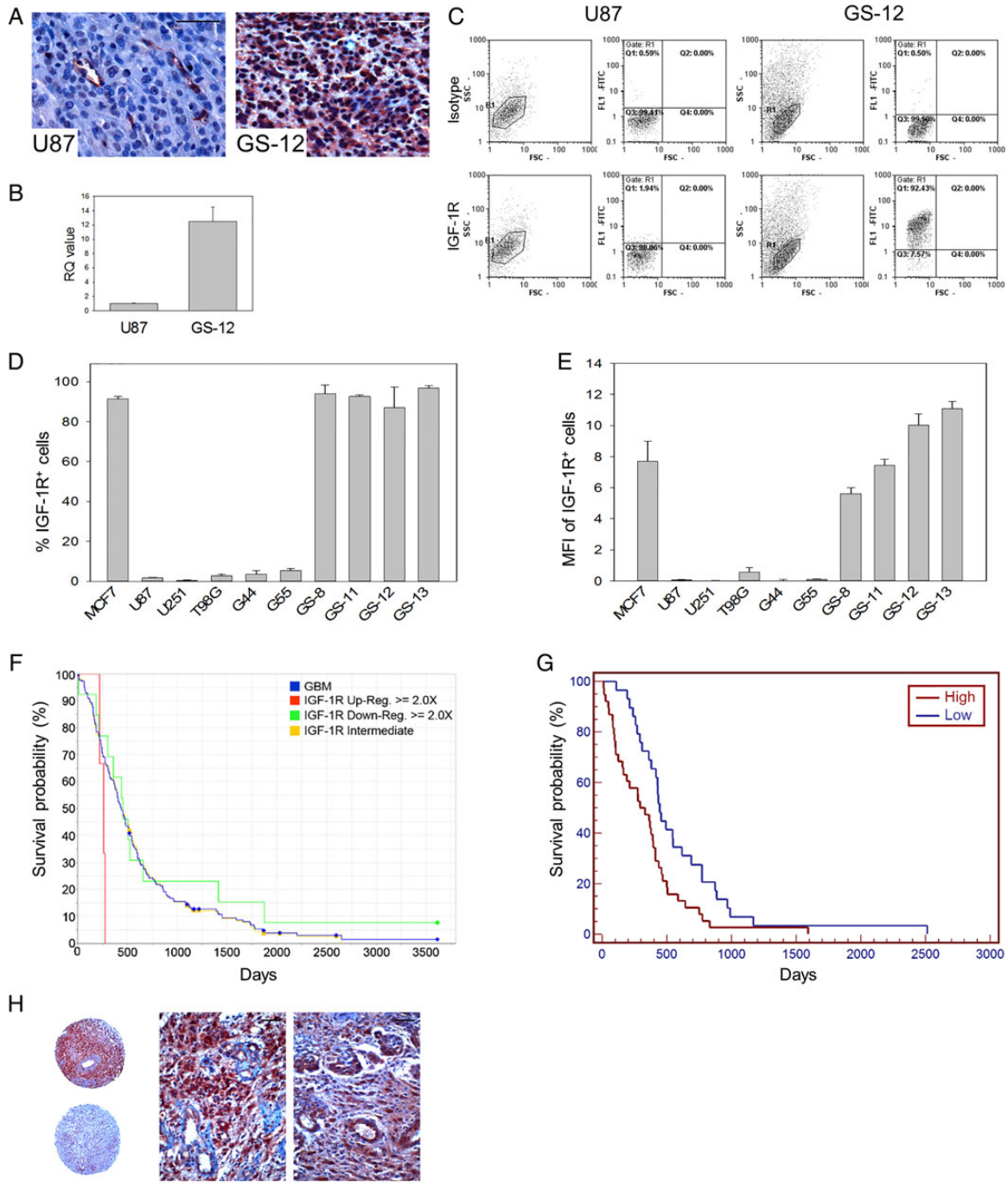

Fig. 2. IGF-1R expression in xenograft tumors, glioma cell lines, and human glioblastomas. (A) Immunohistochemistry for IGF-1R in U87 and GS-12 xenograft tumors. FITC, fluorescein isothiocyanate. (B) Relative quantity (RQ) of IGF-1R transcripts in cell lines analyzed by quantitative PCR. (C) Representative flow cytometry analysis and (D) flow cytometry for IGF-1R on a spectrum of glioma cell lines and MCF7 cells (control). Values are means of quadruplicate determinations ± SD. (E) Mean fluorescence intensity (MFI) per cell. (F) REMBRANDT database analysis. Survival of glioblastoma patients ($N = 181$) with upregulation of IGF-1R (red) was significantly shorter than survival of patients with receptor downregulation (green) ($P = .042$). (G) Analysis of a glioblastoma TMA. Survival of patients with high intratumoral IGF-1R immunoreactivity ($N = 38$, median: 442 d) was shorter than survival of patients with low expression ($N = 29$, median: 311 d) ($P = .011$). (H) Representative examples of TMA samples with high versus low IGF-1R expression (upper vs lower circular sample, left), and tissue sections showing IGF-1R immunoreactivity on tumor cells as well as vascular endothelial cells (right).

determined the receptor status in both cell lines. Quantitative PCR analysis revealed that GS-12 cells expressed >10-fold higher IGF-1R mRNA levels than U87 cells (Fig. 2B). To assess receptor protein levels, we performed flow cytometric analyses and extended these also to additional cell lines. All GS cell lines contained between 87.0% and 97.0% of IGF-1R–expressing

(IGF-1R+) cells, similar to MCF7 cells, which served as positive control, whereas U87 as well as other conventional adherent glioblastoma cell lines (G44, G55, T98G, U251) contained only between 0.3% and 5.3% IGF-1R+ cells (Fig. 2C and D). Furthermore, the mean fluorescence intensity per cell was on average 2.5-fold stronger on GS cells, indicating a higher receptor number per cell (Fig. 2E). We further analyzed whether cell lines expressed IGF-1R ligands. Western blotting and quantitative PCR of cell lysates and supernatants showed that neither IGF-1 nor IGF-2 was produced by U87 or GS-12 cells, indicating no significant autocrine stimulation (Supplementary Fig. S1).

To assess the clinical relevance of IGF-1R expression in human glioblastomas, we interrogated the Repository of Molecular Brain Neoplasia Data (REMBRANDT) of the National Cancer Institute. This analysis showed that glioblastoma patients with upregulation of IGF-1R carry a significantly worse prognosis than patients with relative downregulation of IGF-1R ($P = .0417$; Fig. 2F). Furthermore, we immunostained human glioblastoma sections and a TMA[22] and confirmed the inverse association between IGF-1R expression levels and survival at the protein level ($P = .011$; Fig. 2G). In addition to glioblastoma cells, intratumoral vascular endothelial cells also displayed IGF-1R immunoreactivity (Fig. 2H).

In vitro Effects of Insulin-like Growth Factor 1 Receptor Ligands and IMC-A12 on Glioblastoma Cells

To analyze the functional relevance of the IGF-1R system in GS-12 and U87 cells, IGF-1, IGF-2, and IMC-A12 were tested for their ability to stimulate or inhibit cell proliferation, migration, and apoptosis. First, U87 cells were incubated with IGF-1 or IGF-2 using either serum-free conditions or 1% FCS for 4 days. Neither ligand stimulated the proliferation of U87 cells (Fig. 3A and data not shown). Moreover, IMC-A12 had no effect on growth stimulation of U87 cells by FCS (10%), which contains both IGF-1 and IGF-2 (Fig. 3A). In contrast, proliferation of GS-12 cells was strongly stimulated by IGF-1 and IGF-2, with a maximum increase of 83.2% at 2 nM IGF-1 and of 108.9% at 5 nM IGF-2 after 8 days of incubation (Fig. 3B). Ligand-induced proliferation could completely be blocked by IMC-A12 at concentrations between 25 nM and 200 nM (Fig. 3B), indicating that growth of GS-12 cells but not U87 cells can be driven by paracrine IGF-1R activation.

Second, the relevance of IGF-1R for cell motility was tested using modified Boyden chamber migration assays. Neither IGF-1 nor IGF-2 had a significant chemotactic effect on U87 cells (Fig. 4A). IGF-2 stimulated the migration of GS-12 cells

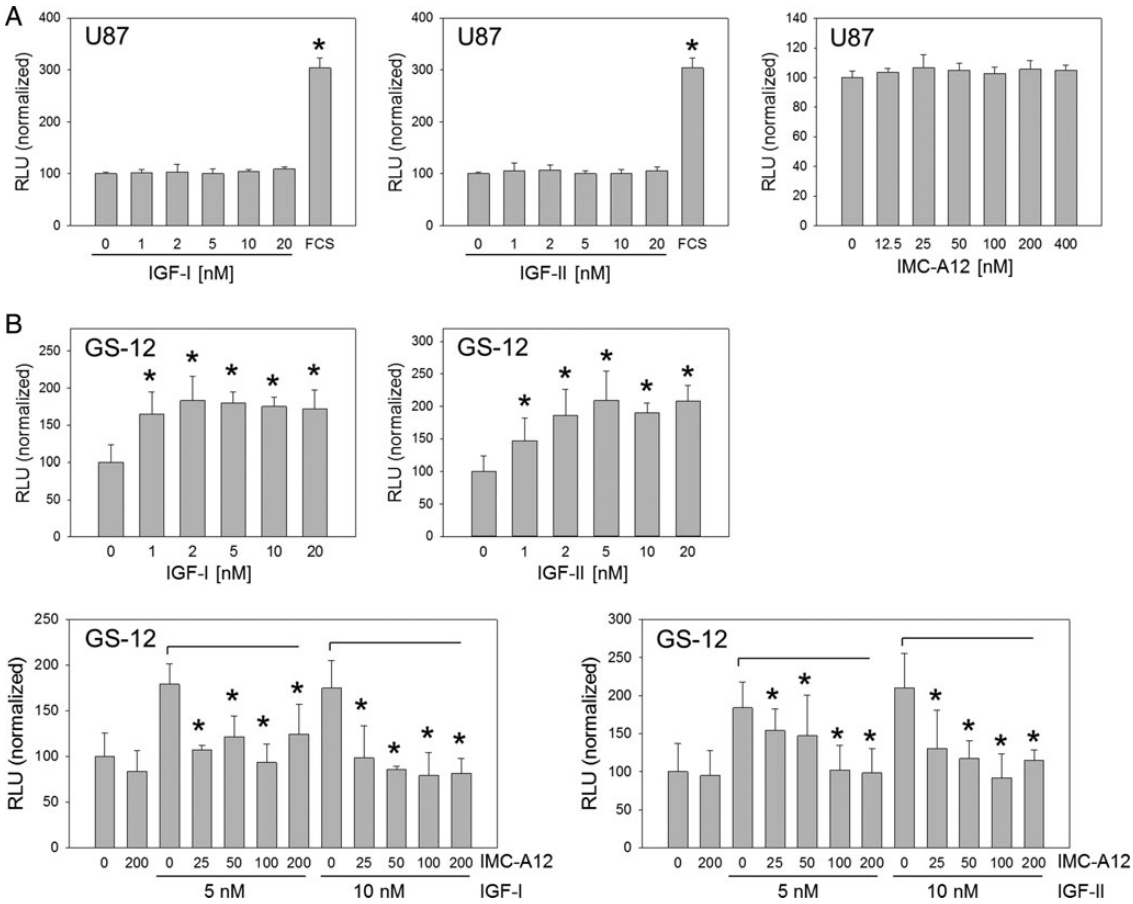

Fig. 3. Glioma cell proliferation in response to IGF-1, IGF-2, and IMC-A12. Serum-starved U87 cells or GS-12 cells (with 4 nM insulin) were incubated over 4 or 8 days, respectively, with IGF-1, IGF-2, IMC-A12, or 10% FCS (control). (A) Neither ligand nor IMC-A12 had an effect on U87 cell proliferation. RLU, relative luminescence units. (B) IGF-1 and IGF-2 stimulated GS-12 proliferation, and mitogenic effects were blocked by IMC-A12. Values are means ± SD of quintuplicate determinations. Asterisks indicate significant stimulation or inhibition ($P < .05$).

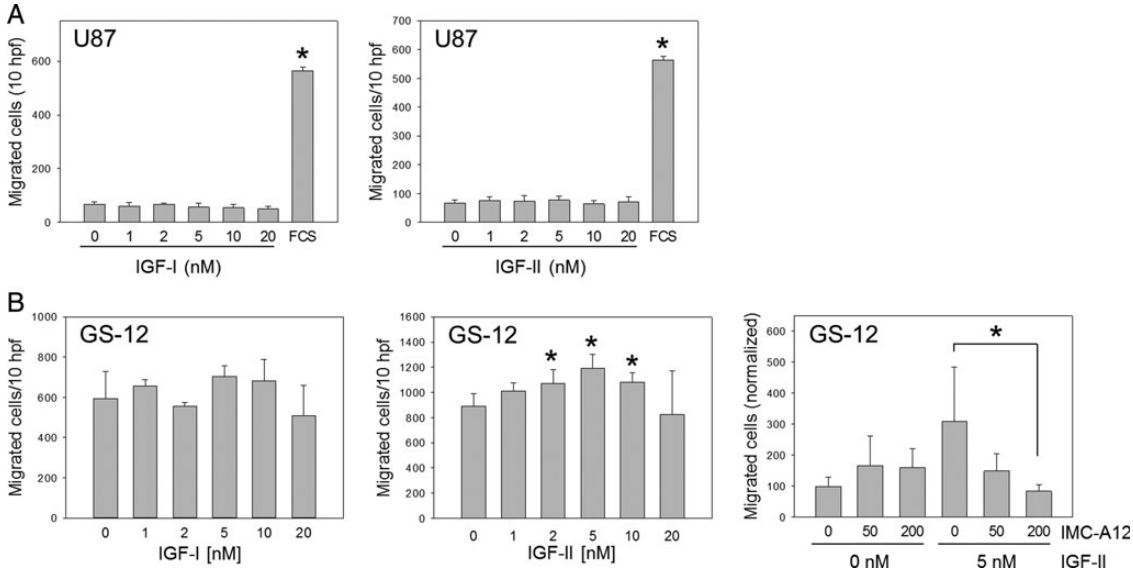

Fig. 4. Glioma cell migration in response to IGF-1, IGF-2, and IMC-A12. Chemotaxis was analyzed using modified Boyden chamber assays. After 6 h (U87) or 20 h (GS-12) of incubation, migrated cells were counted in 10 high power fields (hpf). (A) U87 cell migration was not stimulated by IGF-1 or IGF-2 (10% FCS: control). (B) IGF-2 stimulated GS-12 cell migration, and IMC-A12 inhibited motogenic stimulation. Values are means ± SD of quintuplicate determinations. Asterisks indicate significant stimulation or inhibition ($P < .05$).

with a maximum increase of 34.0% at 5 nM, whereas IGF-1 had no effect. The motogenic effect of IGF-2 was completely blocked by coaddition of IMC-A12 at 200 nM (Fig. 4B).

Third, since another aspect of IGF-1R function is to protect tumor cells from apoptosis, we tested whether IMC-A12 enhanced the rate of apoptosis. Whereas IMC-A12 did not affect apoptosis of U87 cells, a 2.0-fold increase in the proportion of apoptotic GS-12 cells was observed at 200 nM IMC-A12 (Fig. 5).

Last, we tested whether IMC-A12 inhibits ligand-induced activation of IGF-1R in GS-12 cells. IGF-1 and IGF-2 strongly stimulated IGF-1R phosphorylation, and pre-incubation with IMC-A12 inhibited receptor activation (Fig. 6). Furthermore, IMC-A12 induced downregulation of IGF-1R, consistent with its reported capacity to promote receptor internalization and degradation.[14,15]

Discussion

We have shown that local treatment with the IMC-A12 antibody strongly inhibits glioblastoma growth in 2 different orthotopic glioblastoma models, one mimicking the solid, angiogenesis-dependent tumor core component, the other reflecting the diffusely invasive angiogenesis-independent periphery present in human glioblastomas. Treatment was well tolerated, and no signs of toxicity were observed. We chose convection-enhanced delivery (CED) as the route of application of IMC-A12 in order to maximize efficacy, since this technique is currently the most efficient route of application for any drug that, due to high molecular weight or poor permeability, needs to bypass the blood–brain barrier. Promising clinical trials have been completed using CED to deliver cytostatic drugs or toxin conjugates into gliomas.[28,29] Maximization of intracerebral drug distribution also in areas distant from the tumor injection site by CED was particularly relevant in our GS-12 xenograft model, in which widespread brain invasion is a prominent feature.

Interestingly, we identified different mechanisms of tumor growth inhibition in the 2 models. Whereas inhibition of GS-12 tumor progression was apparently mediated by direct effects of IMC-A12 on the tumor cells, growth inhibition of U87 tumors was achieved by an anti-angiogenic effect. These differences in direct tumor cell responsiveness are linked to different receptor expression levels. While U87 cultures contained <2% of IGF-1R+ cells, about 90% of GS-12 cells expressed IGF-1R, and the receptor density per cell was considerably higher. IGF-1R expression on GS-12 cells was in the order of that on MCF7 cells, a breast cancer line that was previously shown to possess a high IGF-1R density and be exquisitely responsive to IGF-1R blockade.[14] Notably, all other GS cell lines analyzed also contained >80% of IGF-1R+ cells, whereas additional conventional adherent glioblastoma cell lines contained <6% positive cells. These findings suggest that neurosphere culture conditions, which preserve the transcriptional phenotype of human glioblastomas better than serum culture conditions and maintain invasive capacities of cell lines upon xenotransplantation,[19] also retain a higher level of IGF-1R expression.

Histological analysis of GS-12 tumors revealed that total tumor burden was reduced by 50.3% in IMC-A12–treated animals, and growth inhibition was apparently due to a direct antiproliferative effect on tumor cells. Consistent with our histological observations, we found that in vitro proliferation of GS-12 cells was strongly stimulated by IGF-1 as well as IGF-2, and mitogenic effects were abrogated by IMC-A12. In situ, we further observed that tumor cell burden was reduced more markedly in areas distant from the site of cell injection than in central tumor regions, suggesting that inhibition of tumor cell invasion into remote brain areas contributed to reduced

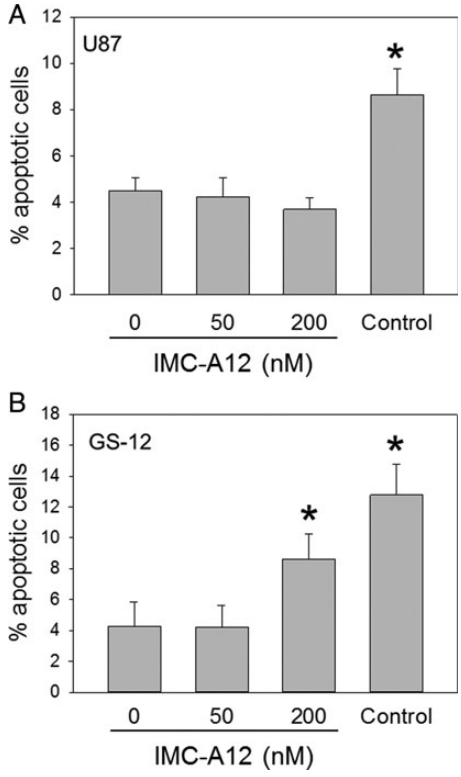

Fig. 5. Glioma cell apoptosis in response to IMC-A12. Apoptotic death was determined by flow cytometry for annexin V. The proportion of apoptotic GS-12 cells but not U87 cells was increased after 72 h of IMC-A12 (200 nM) treatment. Etoposide (25 μM) served as positive control. Values are means ± SD of quintuplicate determinations. Asterisks indicate significance ($P < .05$).

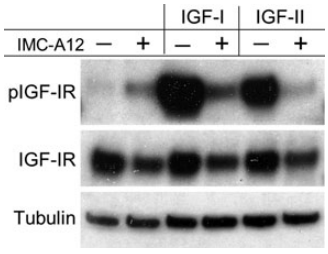

Fig. 6. Inhibition of IGF-1R activation by IMC-A12. GS-12 cells were cultured in low insulin medium and pre-incubated with 200 nM IMC-A12 for 2 h prior to stimulation with 10 nM IGF-1 or IGF-2 for 10 min. IMC-A12 inhibited ligand-induced IGF-1R phosphorylation and reduced the level of detectable receptor.

tumor progression in vivo. However, it is impossible with the currently available histological techniques to differentiate to what degree distant tissue infiltration is due to actual tumor cell migration/invasion or rather to continuous diffuse growth of the proliferating tumor. In vitro, we found that IGF-2 strongly stimulated GS-12 cell migration, and motogenic effects were abrogated by IMC-A12, indicating that IGF-1R activation by IGF-2 contributes to cell motility. Notably, IGF-1 was not chemotactic, suggesting that IGF-2–induced motility might in part also be mediated through the IR, which can bind IGF-2 but not IGF-1. A previous gene expression profiling study showed that the IR gene is expressed in GS-12 cells (Gene Expression Omnibus database: GSE23806).[19] Taken together, our findings support the notion that an anti-invasive component is part of the antitumor activity of IMC-A12 in vivo.

IMC-A12 was demonstrated to inhibit growth of various tumor cell types in vitro and in vivo, including breast, prostate, lung, and colon cancer.[15] Mechanistically, growth inhibition by IMC-A12 has largely been attributed to antiproliferative and pro-apoptotic effects; and in glioma cells, IGF-1R signaling can mediate resistance to radiation-induced apoptosis.[8] We observed a significant pro-apoptotic effect of IMC-A12 on GS-12 cells in vitro as well as a moderate increase in apoptosis in IMC-A12–treated xenografts, indicating that pro-apoptotic effects contributed to growth inhibition of GS-12 tumors. As GS-12 cells lack IGF-1 and IGF-2 expression but IGF-1R is nevertheless weakly activated in unstimulated cells, the IMC-A12–induced increase in apoptosis could be due to blockade of IGF-1R or hybrid receptor activation by low levels of insulin present in the medium.

While the IGF-1R system is clearly relevant for GS-12 cell proliferation and migration, we could detect neither any mitogenic or motogenic effect of IGF-1 or IGF-2 on U87 cells nor any pro-apoptotic effect of IMC-A12 in vitro. This lack of any functional effect is consistent with the low IGF-1R expression level in U87 cells. Several previous studies analyzed IGF-1R expression in various glioma cell lines, and while some lines expressed relatively high receptor levels (eg, C6, LN-18, LN-229), most studies detected only comparatively low IGF-1R expression in U87 cells.[7,13,23,25] Nevertheless, a few in vivo studies attempted to antagonize the IGF/IGF-1R system in U87 cells. Most studies, however, used nonspecific inhibitors such as picropodophyllin and NVP-TAE226, which target also other molecules or pathways unrelated to IGF-1R.[13,25] One study targeted IGF-1R specifically by using another anti–IGF-1R antibody, αIR3.[26] The α-IR3 antibody reduced the total number of tumors that formed subcutaneously but apparently not their size, indicating that the antibody primarily inhibited tumor initiation. In our orthotopic U87 model, we initiated treatment after 7 days when tumors were well established to exclude that we merely inhibit tumor initiation rather than evaluating treatment efficacy. Under our conditions, inhibition of tumor angiogenesis was the main visible effect that could explain the observed growth inhibition. The moderate reduction of tumor cell proliferation is most likely an indirect consequence of the reduced vascular supply in treated tumors. Angiogenesis inhibition by IMC-A12 has also previously been observed in multiple myeloma xenografts and was attributed to suppression of VEGF secretion from myeloma cells.[30] We therefore tested whether U87 cells increased the expression of VEGF in response to incubation with IGF-1 or IGF-2, but found no effect (unpublished observation). Therefore, anti-angiogenic effects on U87 tumors are more likely mediated by a direct action of IMC-A12 on endothelial cells, consistent with studies showing that IGF-1 and IGF-2 can stimulate endothelial cell migration and tube formation.[31,32]

Neither U87 nor GS-12 cells displayed autocrine secretion of IGF-1 or IGF-2. Hence, activation of IGF-1R in GS-12 xenografts was most likely due to paracrine stimulation. IGF-1 and IGF-2 are produced by numerous cell types in the body and circulate in ~1000-fold higher concentrations than most other known

peptide hormones.[1,2] In several rodent models, the growth of IGF-1R–expressing cancers was demonstrated to be stimulated by circulating IGF-1 produced remotely from the tumor.[15,33] In humans, high levels of circulating IGF-1 are associated with an increased risk of developing colon, breast, prostate, and lung cancer as well as low-grade glioma.[1,34] These findings suggest that paracrine activation by murine IGF-1 or IGF-2, which can bind human IGF-1R, drives growth of GS-12 tumors, and this mechanism is amenable to IMC-A12 targeting.

While most human glioma cell lines lack IGF expression, a subgroup of human gliomas were reported to express IGF-1 and/or IGF-2, indicating a possible autocrine component.[3–6,24,35] In human astrocytomas, IGF-1, IGF binding proteins, and IGF-1R were demonstrated to be expressed at levels higher than in normal adult brain. Hirano and colleagues[5] examined IGF-1 and IGF-1R expression in astrocytomas by immunohistochemistry and in situ hybridization and showed that the proportion of tumor cells expressing IGF-1 and IGF-1R correlated with the Ki-67 index. Strong expression of IGF-2 mRNA and protein was also detected in astrocytoma cells but not in normal brain.[3] Among glioblastomas that lack epidermal growth factor receptor amplification or overexpression, a subset of tumors was identified that strongly overexpress IGF-2, associated with loss of phosphatase and tensin homolog, high proliferation, and poor survival.[35] Furthermore, the clinical relevance of the IGF-1R system is underlined by our REMBRANDT and TMA analysis, showing that glioblastoma patients with upregulation of IGF-1R carry a significantly worse prognosis.

To conclude, we showed that glioblastoma growth in vivo can be inhibited by interstitial delivery of the IMC-A12 antibody, and growth inhibition can be achieved by direct effects on the tumor cells as well as indirect effects on the vasculature. Given the prognostic relevance of IGF-1R upregulation in glioblastoma patients, CED of IMC-A12 appears to be a promising strategy to inhibit glioblastoma progression and effectively complement the currently available conventional treatment options.

Supplementary Materials and Methods

Supplementary material is available at *Neuro-Oncology Journal* online (http://neuro-oncology.oxfordjournals.org/).

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