Inhibition of Glioblastoma Angiogenesis and Invasion by Combined Treatments Directed Against Vascular Endothelial Growth Factor Receptor-2, Epidermal Growth Factor Receptor, and Vascular Endothelial-Cadherin

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Abstract

Purpose: Inhibition of angiogenesis can influence tumor cell invasion and metastasis. We previously showed that blockade of vascular endothelial growth factor receptor-2 (VEGFR-2) with the monoclonal antibody DC101 inhibited intracerebral glioblastoma growth but caused increased tumor cell invasion along the preexistent vasculature. In the present study, we attempted to inhibit glioma cell invasion using a monoclonal antibody against the epidermal growth factor receptor (EGFR), which in the context of human glioblastomas, has been implicated in tumor cell invasion. In addition, we analyzed whether blockade of vascular endothelial (VE)-cadherin as a different antiangiogenic target could also inhibit glioblastoma angiogenesis and growth.

Experimental Designs: Nude mice who received intracerebral glioblastoma xenografts were treated using monoclonal antibodies against VEGFR-2 (DC101), EGFR (C225), and VE-cadherin (E4G10) either alone or in different combinations.

Results: Increased tumor cell invasion provoked by DC101 monotherapy was inhibited by 50% to 66% by combined treatment with C225 and DC101. C225 inhibited glioblastoma cell migration in vitro, but had no effect on the volume of the main tumor mass or on tumor cell proliferation or apoptosis in vivo, either alone or in combination with DC101. The anti-VE-cadherin monoclonal antibody E4G10 was a weaker inhibitor of tumor angiogenesis and growth than DC101, and also caused a weaker increase in tumor cell invasion.

Conclusions: Inhibition of angiogenesis achieved by blocking either VEGFR-2 or VE-cadherin can cause increased glioma cell invasion in an orthotopic model. Increased tumor cell invasion induced by potent inhibition of angiogenesis with DC101 could be inhibited by simultaneous blockade of EGFR.

Glioblastomas are densely vascularized tumors. Antiangiogenic therapy for glioblastomas is considered promising, and various preclinical experimental strategies targeting neovascularization have proven effective in vivo. We previously showed that systemic therapy with an antibody against vascular endothelial growth factor receptor-2 (VEGFR-2) inhibited glioblastoma growth by ~80% (1). However, we observed a strikingly more invasive growth pattern in tumors treated with DC101. Invasion occurred primarily along preexistent host vessels which were often cuffed over long distances by tumor cells.

The phenomenon that tumors which arise in a well-vascularized tissue environment can recruit their vascular supply from preexistent vessels and grow despite the absence of neovascularization has been termed vessel cooption (2). This phenomenon is particularly pertinent to gliomas, as the brain is a very well vascularized organ, and malignant glioma cells can spread diffusely without necessarily requiring neovascularization (3, 4). From our previous findings, we conclude that in a therapeutic situation in which angiogenesis is inhibited, the two pathways can become alternatives, so that blockade of neovascularization favors vessel cooption as a compensatory mechanism (1, 5). Several other recent studies provide additional evidence that inhibition of tumor angiogenesis can modulate patterns of tumor invasion (6–8).

In the present study, we attempted to inhibit the increased glioma cell invasion observed with DC101 therapy by combined treatment with an antibody against the epidermal growth factor receptor (EGFR), which in the context of human gliomas, acts as a strong stimulator of tumor cell migration and invasion (9–11). In addition, we analyzed whether blocking another vascular target, vascular endothelial...
VE-cadherin would have similar effects as DC101 on tumor angiogenesis, growth, and invasive morphology. As our previous study showed that DC101 did not abrogate glioma angiogenesis completely (1), we further analyzed whether the combination of VEGFR-2 and VE-cadherin blockade would have synergistic effects and could augment the antiangiogenic and antitumor activity over that achievable with DC101 alone.

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Fig. 1. Comparisons between tumors treated with either PBS, DC101, C225, E4G10, or combinations of DC101 and E4G10. Treatment was initiated either on day 1 after tumor cell injections (A–E), or on day 6 (F–J). *, significance (P < 0.05), values obtained for PBS controls, unless indicated otherwise.
Materials and Methods

Antibodies. All antibodies used for in vivo experiments were provided by ImClone Systems (New York, NY). DC101 is a rat monoclonal antibody against mouse VEGFR-2 (12). C225 is a mouse/human chimeric antibody against human EGFR, in which the constant region of a mouse monoclonal antibody was chimerized with human IgG1 (13). E4G10 is a rat monoclonal antibody against murine VE-cadherin (14). The polyclonal antibody against von Willebrand factor and the monoclonal antibody MIB-1 against the Ki-67 antigen were obtained from Dako (Hamburg, Germany). Rabbit polyclonal anti-cleaved caspase-3 was from Cell Signaling Technology (Beverly, MA), and polyclonal rabbit anti-human EGFR antibody from Sigma (St. Louis, MO).

Orthotopic glioma model. Intracerebral glioblastoma xenografts were established as described previously (1). Briefly, the G55 human glioblastoma cell line was injected into the caudate/putamen of female severe combined immunodeficiency mice (C.B.-17 scid/scid) aged 6 to 8 weeks (7 × 10⁶ cells per injection). Treatment was initiated on the day after tumor cell injection (day 1). Six different treatment groups were formed, comprising 12 animals each. Treatment consisted of i.p. injections of either DC101, C225, E4G10 or PBS alone, or DC101 in combination with C225 or with E4G10. Antibody doses were selected based on effectivity and toxicity studies done at ImClone Systems, and were 800 μg per injection for DC101 or 1 mg for C225 and E4G10. Antibodies were injected every 3 days until day 16 when animals were sacrificed using CO₂ or perfusion fixation with 4% glutaraldehyde. The experiment was repeated with a modified schedule, in which the same types of treatment were initiated on day 6 when tumors were already established instead of on day 1.

Determination of tumor size. Mouse brains were fixed in formalin, bisected coronally, and embedded in paraffin. The maximum cross-sectional area of the intracranial glioblastoma xenografts was determined on H&E-stained serial sections by computer-assisted image

Fig. 2. Histologic appearance of treated tumors and controls. H&E-stained sections show the extent and morphology of tumors treated with PBS (A), DC101 (B), C225 (C), DC101 combined with C225 (D), and DC101 combined with E4G10 (E). Insets, examples of tumor surfaces and adjacent parenchyma; DC101-treated tumors are surrounded by numerous smaller satellite tumors (F) which is in contrast to tumors cotreated with C225 (G). Staining for von Willebrand factor typically reveals blood vessels in the center of peritumoral satellites (F and G), and occasional longitudinal sections of blood vessels show that the vasculature is often cuffed over considerable distance by tumor cells (H). von Willebrand factor staining further reveals a strikingly higher blood vessel density in PBS control tumors (I) than in DC101-treated tumors (J). Semithin sections stained with toluidin show markedly dilated vessels at the edge of control tumors (K), in contrast to almost complete absence of dilated vessels in DC101-treated tumors (L). MIH-1 immunohistochemistry reveals a higher proliferative activity in PBS control tumors (M) than in DC101-treated tumors (N). The fraction of apoptotic tumors cells in PBS control tumors, as detected by staining for cleaved caspase-3 (O), is lower than in DC101-treated tumors (P).
analysis. The tumor volume was estimated using the formula: volume = \(\text{area} \times \text{height}\). Immunohistochemical and ultrastructural analysis. Paraffin sections were immunostained according to standard protocols. To detect microvessels, sections were stained with an antibody against von Willebrand factor (VWF; 1:200). Vessel density was determined by counting the number of stained vessels in three high-power fields (0.031 mm\(^2\)) in the most densely vascularized “hotspot” area. To analyze the proliferative activity of the tumor cells, sections were stained with the MIB-1 antibody (1:50) against the Ki-67 antigen. The percentage of MIB-1-positive nuclei was determined by counting immunoreactive tumor cell nuclei in three high-power fields in the most actively proliferating tumor area. To detect apoptotic cells, paraffin sections were stained using an antibody against cleaved caspase-3 (1:200). The percentage of cells expressing caspase-3 was determined by counting immunoreactive cells in five high-power fields, avoiding necrotic tumor areas. To prepare semithin and ultrathin sections, small tissue blocks from perfusion-fixed brains were embedded in Epon and processed for light and electron microscopy according to routine protocols.

Western blot analysis. G55 cells were lysed in PBS containing 1% SDS. Total protein was precipitated using methanol and chloroform. Pellets were resuspended in loading buffer containing \(\beta\)-mercaptoethanol. Proteins (40 μg) were separated by SDS-PAGE on 8% gels, blotted onto polyvinylidene difluoride membranes, and probed with a polyclonal rabbit anti-human EGFR antibody.

Modified Boyden chamber migration assay. The effects of transforming growth factor-α (TGFα), conditioned medium from G55 glioblastoma cells, C225, DC101, and E4G10 on the motility of G55 glioblastoma cells were analyzed using a modified Boyden chamber assay as described previously (11). Briefly, TGFα (Peprotech, Rocky Hill, NJ) or serum-free conditioned medium concentrated 15-fold, and/or C225 were 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 coated with Vitrogen 100 (Collagen, Fremont, CA). G55 cells (1.5 \(\times\) 10\(^4\)) were seeded into the upper wells. After a 5-hour incubation at 37°C, nuclei of migrated cells were stained and counted in 10 high-power fields.

Methods of data analysis. Differences between tumor volumes, numbers of satellite tumor nodules, vessel densities, proliferation, and apoptotic indices were analyzed using the unpaired t test or Mann-Whitney rank-sum test.

Results

Tumor growth in vivo. All animals injected with G55 human glioblastoma cells developed tumors. In the first experiment, treatment was initiated on day 1 after tumor cell injection; in a second modified repeat experiment, treatment was started on day 6 when tumors were already established. Treatment consisted of either DC101, C225, E4G10 or PBS alone, or DC101 in combination with C225 or E4G10. In both experiments, significant inhibition of tumor growth was obtained by DC101 and by combined treatments which included DC101 as one component (Figs. 1A,F and 2A-E). When treatment was initiated on day 1, the mean tumor volume in mice treated with DC101 was reduced by 75.1% compared with animals treated with PBS; when treatment was initiated on day 6, tumor volume was reduced by 73.3%. Significant inhibition of tumor growth was also obtained with E4G10, which caused a 62% reduction in tumor volume in experiment 1, but displayed only a nonsignificant tendency of 36.8% growth inhibition when treatment was initiated after 6 days of tumor growth (P = 0.075). C225 monotherapy had no effect on tumor volume. Combined treatment of DC101 with either C225 or E4G10 resulted in a similar degree of inhibition as DC101 alone. In the control groups and the C225-treated groups between 25% and 42% of the animals had developed weight loss when the experiments were terminated, whereas none of the animals in the other treatment groups had developed symptoms.

Tumor morphology. A strikingly more invasive growth pattern was observed for tumors treated with DC101 compared with PBS controls. Tumors in DC101-treated animals were usually surrounded by numerous small satellite tumors (Fig. 2B). Most of these satellites contained discernible central vessel cores, which represented cross-sections of blood vessels cuffed by invading tumors cells (Fig. 2F-H). Quantification of these satellites revealed that the mean satellite frequency was increased by 88.2% in DC101-treated mice compared with PBS controls in the first experiment, and was increased by 204.9% in the second experiment (Fig. 1B and G).

The increase in satellite number in DC101-treated mice confirms our previous observations (1), which had led us to conclude that for gliomas, antiangiogenic therapy should ideally be combined with antiinvasive therapy. Expression of EGFR by glioma cells has mainly been associated with mediating tumor cell migration and invasion (10). Therefore, we analyzed whether antiinvasive therapy targeting EGFR could attenuate the increased tumor cell invasion caused by DC101 treatment. Indeed, combination of DC101 with C225 led to a significant decrease in the number of peritumoral satellites compared with DC101 monotherapy (Figs. 1B,G and 2D). In the first experiment, cotreatment with C225 reduced the mean satellite number by 65.9% compared with DC101 alone; in the second experiment, the reduction was 50.1%. The satellite number observed with C225 monotherapy did not differ significantly from that in PBS controls.

Antangiogenic treatment with E4G10 also had a stimulatory effect on the number of peritumoral satellites compared with PBS controls with an increase in satellite number of 42.2% in the first experiment, but only a nonsignificant similar tendency in the second. When E4G10 was combined with DC101, satellite numbers in both experiments did not differ significantly from those observed for DC101 monotherapy.

Tumor microvessel density. In DC101-treated mice, intratumoral microvessel density was significantly reduced compared with PBS controls (Figs. 1C, H and 2I, J). The reduction was 41.1% in the first experiment and 41.6% in the second. Semithin sections prepared from perfusion-fixed tumors revealed markedly dilated blood vessels around the tumor mass in control tumors (Fig. 2K), but far fewer and less dilated peritumoral vessels in DC101-treated tumors (Fig. 2L). Ultrastructurally, intratumoral vessels in DC101-treated tumors were indistinguishable from those in PBS controls (data not shown). When treatment was initiated on day 1, E4G10 treatment also reduced the intratumoral microvessel density by 28.0%, but no significant effect was detectable in the second experiment. C225 had no effect on microvessel density compared with PBS. The effects of either C225 or E4G10 in combination with DC101 were similar to those obtained by DC101 treatment alone.

Tumor cell proliferation. In DC101-treated tumors, proliferative activity was reduced by 45.7% when treatment was started on day 1, and by 39.1% when treatment was started...
on day 6 (Figs. 1D, I and 2M, N). In the first experiment, E4G10 treatment also caused a significant 26.7% reduction in tumor cell proliferation, whereas no significant reduction was found when treatment was initiated on day 6. The proliferative activities in tumors treated with combinations of DC101 and either C225 or E4G10 were similar to those in tumors treated with DC101 alone.

**Tumor cell apoptosis.** DC101-treated tumors displayed a 77.7% and 82.1% increase in the percentage of apoptotic tumor cells compared with PBS controls in experiments 1 and 2, respectively (Figs. 1EJ and 2OP). An increased apoptotic rate was also observed in tumors of E4G10-treated animals; in the first experiment, the fraction of apoptotic tumor cells was increased by 40.4% compared with PBS controls, however, in the second experiment, no significant effect was found. Apoptotic rates in animals receiving combined treatments of DC101 with either C225 or E4G10 were similar to those in tumors treated with DC101 alone.

**C225 inhibition of glioma cell migration in vitro.** To analyze whether C225 inhibits TGFα-induced migration of G55 glioblastoma cells, modified Boyden chamber chemotaxis assays were done. TGFα induced a maximally 5.0-fold stimulation of G55 migration at 1 nmol/L (Fig. 3A). The effect of TGFα was inhibited in a dose-dependent fashion by coaddition of C225 with complete inhibition at concentrations ≥100 ng/mL C225. In addition, C225 inhibited migration of G55 cells induced by concentrated conditioned medium (Fig. 3B). DC101 had no significant effect on G55 migration induced by TGFα (Fig. 3C) or conditioned medium (Fig. 3D) and did not significantly alter the inhibitory effects on migration observed with C225. E4G10 also had no significant effect on G55 migration, either alone or in combination with DC101 (data not shown). Expression of EGFR by G55 cells was shown by Western blot analysis (Fig. 3E), and expression of TGFα was detected by RT-PCR analysis (data not shown).

**Discussion**

Previous studies showed that antagonization of the VEGF/VEGFR-2 system can inhibit the growth of intracerebral gliomas in rodents and can prolong survival by almost 100% (1, 6). However, there is increasing evidence that in several different tumor models, antiangiogenic therapy can lead to enhanced tumor cell invasion or metastasis (1, 6–8, 15, 16). Orthotopic glioma models showed that antagonization of neovascularization could cause increased tumor cell migration, preferentially along preexistent host vessels (1, 6). Although the exact mechanisms responsible for this increased invasiveness are unknown, it has been speculated that a decreased supply of oxygen and nutrients may act as a stimulus for tumor cell migration. EGFR signaling can strongly stimulate glioblastoma cell migration and invasion (9–11). C225 is a well-characterized and potent EGFR blocking antibody that inhibits receptor autophosphorylation and induces internalization and degradation (17). It was shown that C225 can inhibit glioblastoma growth in vivo and prolong survival of mice bearing intracerebral glioma xenografts by at least 900% (18, 19). Therefore, we chose this agent to attempt inhibition of the compensatory increase in glioma cell invasion observed with antiangiogenic therapy.

The results of this study confirm our previous observation that DC101 treatment inhibits growth of the main tumor mass by >70%, and reduces intratumoral vessel density by >40%, but causes a strong increase in tumor invasion which can be quantified as number of small satellite tumors surrounding the primary mass. Combined treatment of DC101 with C225 reduced the number of satellite tumors by 65.9% when treatment was initiated on day 1 after tumor cell injection, and by 50.1% when it was initiated on day 6. In addition, C225 inhibited TGFα-induced glioma cell migration in vitro, and presumably blocked an autocrine loop of TGFα secreted by tumor cells which stimulates migration. However, C225 did not affect the size of the main tumor mass and had no effect on the proliferative activity or apoptotic rate of the tumor cells in vivo. These findings are in line with previous reports demonstrating that EGFR activation strongly stimulates glioma cell migration and invasion but not necessarily proliferation (9–11). Interestingly, a recent study showed that C225 was only proapoptotic and cytotoxic for glioblastoma cell lines that harbored EGFR gene amplifications, but not for nonamplified cell lines (18). Moreover, it was found that different agents that block EGFR signaling inhibit glioblastoma cell invasion at much lower concentrations than are necessary for growth suppression (10). These findings may explain why in our model using the G55 glioblastoma cell line, which does not contain an EGFR amplification (data not shown), invasion but not proliferation or apoptosis was affected by C225 treatment.

Some clinical studies conducted about a decade ago attempted to block EGFR signaling in glioblastoma patients with less effective and nonhumanized antibodies (20, 21). Although these studies could not demonstrate the beneficial effects of this treatment, they found that the antibodies did accumulate in the tumors and thus crossed the blood-brain barrier (20). One reason why the antibodies were thought to be ineffective was that they did not diffuse far inside the brain. It is possible that in our model, in which tumor cell invasion is particularly strongly associated with the vasculature, this diffusion limit presents less of an obstacle. Interestingly, a recent study showed that small s.c. gliomas were more amenable to growth inhibition by C225 treatment than large ones (19). In addition to diffusion problems which may restrict accessibility for therapeutic agents in more sizeable tumors, specific treatment resistance could also be a greater problem as

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**Fig. 3.** Inhibition of G55 glioblastoma cell migration by C225 in a modified Boyden chamber assay. TGFα in the absence or presence of C225 was added to serum-free medium in the lower wells of the modified Boyden chamber; G55 cells were seeded into the upper wells; after a 5-hour incubation, migrated cells were counted (A). Conditioned medium was collected from G55 cells, concentrated 15-fold, and was added to the lower wells of the chamber with or without coaddition of C225 in concentrations as indicated; G55 cells were seeded into the upper wells and were allowed to migrate for 5 hours (B). TGFα was detected by Western blot analysis (C). Inhibition of cell migration compared with stimulation by TGFα or conditioned medium (D–E). Expression of wild-type EGFR was confirmed by Western blot analysis (F). Values in (A–D) are means ± SE of triplicate determinations. Stimulation of migration by TGFα or conditioned medium was significant in all experiments (A–D). Significant inhibition of cell migration compared with stimulation by TGFα or conditioned medium (B–D). Expression of wild-type EGFR was confirmed by Western blot analysis (E).
large tumors may contain a higher number of potentially resistant tumor cells. It has been shown that glioma cells could become resistant to small molecule inhibitors of EGFR (22, 23), and resistance can principally also occur with C225 treatment (24). Consequently, it is an important goal for future studies to determine whether the antiinvasive effects of C225 in our model can also become subject to resistance.

The other question addressed in this study was whether blockade of VE-cadherin as a different vascular target would have similar effects as blockade of VEGFR-2. We found the inhibitory effect of E4G10 on tumor growth and angiogenesis to be considerably weaker than that of DC101, and to depend on early initiation of treatment. Furthermore, combined treatment with E4G10 and DC101 was not superior to DC101 monotherapy. The relatively small effects of E4G10 confirm the results of various s.c. tumor models, in which blockade of VE-cadherin also turned out to be considerably less effective than blockade of VEGFR-2 (14, 25). E4G10 inhibits VE-cadherin function during angiogenesis, but does not disrupt existing adherens junctions on normal vasculature (14). It is thus possible that E4G10 has no effect on adherens junctions formed during the first 6 days of tumor growth, which may explain why its effect was not strong enough to reach significance in the second experiment. In contrast, DC101 had a similar effect on vessel density regardless of when therapy was initiated, suggesting that blockade of VEGFR-2 may also be deleterious for the maintenance of incompletely stabilized vessels formed during the first 6 days of tumor growth. The effects of VE-cadherin on satellite formation, proliferation, and apoptosis were also weaker than those of DC101, and only reached significance when treatment was initiated on day 1. Stimulation of glioblastoma cell invasion not only by DC101 but also by E4G10, suggests that this phenomenon is not restricted to blockade of VEGFR-2, but occurs as a compensatory mechanism when angiogenesis is efficiently inhibited, independent of the mechanism by which angiogenesis inhibition is achieved.

To conclude, this study shows that antiangiogenic glioblastoma therapy targeting either VEGF-2 or VE-cadherin can inhibit tumor growth, but will increase tumor cell invasion in an orthotopic model. The increased tumor cell invasiveness caused by blockade of VEGFR-2 could be inhibited by simultaneous antagonization of EGFR. Our findings suggest that antagonization of EGFR signaling with a potent humanized monoclonal antibody such as C225 might hold promise as an antiinvasive glioblastoma treatment, which could be particularly beneficial when growth of the main tumor mass is simultaneously inhibited by antiangiogenic therapy.

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References
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