Inhibition of Fibroblast Growth Factor/Fibroblast Growth Factor Receptor Activity in Glioma Cells Impedes Tumor Growth by Both Angiogenesis-dependent and -independent Mechanisms

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ABSTRACT

We undertook a series of systematic studies to address the role of fibroblast growth factor/fibroblast growth factor receptor (FGF/FGFR) activity in tumor growth and angiogenesis. We expressed dominant-negative FGF2R (FGF2R-DN) or FGF1R (FGF1R-DN) in glioma C6 cells by using constitutive or tetracycline-regulated expression systems. Anchorage-independent or independent growth was inhibited in FGF1R-DN-expressing cells. Tumor development after xenografting FGF2R-DN-expressing cells in immunoodeficient mice or after transplantation in rat brain was strongly inhibited. Quantification of microvessels demonstrated a significant decrease in vessel density in tumors derived from FGF1R-DN-expressing cells. Furthermore, in a rabbit corneal assay, the angiogenic response after implantation of FGF1R-DN-expressing cells was decreased. In tumors expressing FGF1R-DN, vascular endothelial growth factor expression was strongly inhibited as compared with control tumor. These results indicate that inhibition of FGF activity may constitute a dominant therapeutic strategy in the treatment of FGF-producing cerebral malignancies and may disrupt both angiogenesis-dependent and -independent signals required for glioma growth and invasion.

INTRODUCTION

FGFs are a large family of regulatory molecules. They have been demonstrated to stimulate growth, survival, and/or differentiation of a number of mesenchyme-derived cells such as fibroblasts, smooth muscle cells, epithelial cells, endothelial cells, and cells derived from the nervous system.

FGFs interact with four prototypes of tyrosine kinase receptors (1). These include FGFR1 (flg), FGFR2 (bek), FGFR3, and FGFR4. These receptors have common features including a cytoplasmic conserved tyrosine kinase domain, a transmembrane domain, and an extracellular ligand binding domain, which may contain two or three immunoglobulin-like domains. A number of splice variants within these different receptor families have been described. Each member of the FGF family preferentially binds to specific receptor splice variants. For instance, FGF-2 or FGF-4 preferentially associates with the IIIc variants, whereas FGF-7 binds to IIIb variants (1).

FGFs and their receptors are thought to be implicated in the development of a number of malignant tumors such as melanoma (3, 4), glioma (5). Morisson et al. (5) reported that glioma cell growth can be inhibited by antisense oligonucleotides to FGF-2. Furthermore, Wang and Becker (4) demonstrated that antisense targeting of FGF-2 or FGF1R in human melanoma inhibits tumor growth. Moreover, a FGF-2 binding protein (FGF-BP) that mobilizes FGF-2 from the extracellular matrix was expressed after malignant progression in carcinoma. Depletion of human squamous cell carcinoma (SCC ME-180) and colon carcinoma (LS174T) cell lines of their endogenous FGF-BP by targeting with specific ribozymes leads to inhibition of tumor cell growth in vitro and in vivo (6). These results suggest that FGF is implicated in tumor growth in vitro and in vivo. However, antisense strategies are questionable because of inhibition of both nuclear and extracellular FGF isoforms. Furthermore, the strategies described above did not clearly answer the question whether FGF signaling is implicated at the level of the tumor cell or at the level of the surrounding stroma, and whether FGF receptor deregulation has any incidence on tumor-stroma cell interactions such as angiogenesis. Receptor expression has also been demonstrated to be modulated during malignant progression. Low-grade astrocytoma or normal white matter exhibit FGFR2 and only low levels of FGFR1. Malignant astrocytomas acquire FGFR1-β (1 immunoglobulin loop form) expression and loose FGFR2 expression (7). This may also contribute to the growth advantage of malignant cells.

We undertook a series of systematic studies to clearly evaluate the involvement of FGF/FGFR activity in glioma development. We chose to disrupt FGF activity at the cell surface level by using a FGF1R-DN strategy (8–10). Furthermore, we used a tetracycline-regulated expression system to control FGF2R-DN and FGF1R-DN in vitro and in vivo. We herein present evidence that disruption of FGF activity leads to the inhibition of glioma growth by both angiogenesis-dependent and -independent mechanisms and may therefore constitute a dominant strategy for the treatment of FGF-producing cerebral tumors.

MATERIALS AND METHODS

Cells. Rat C6 glioma cells (kindly donated by Dr. Paul Canioni, University Bordeaux II, Bordeaux, France) were grown in DMEM (Life Technologies, Cergy-Pontoise, France) containing 7.5% FCS (Life Technologies) and antibiotics in a 5% CO2 atmosphere.

Transfection of C6 Glioma Cells. For stable constitutive expression of FGF2R-DN in C6 glioma cells, C6 glioma cells were cotransfected with the pRK5 expression vector containing human FGF2R-DN cDNA (tyrosine kinase domain deleted, 3 immunoglobulin-like loop form, IIIc splice variant, kindly donated by Dr. Joseph Schlessinger, Department of Pharmacology, New York University Medical Center, New York, NY) and the pCEP4 vector containing a hygromycin-resistant gene at a ratio of 1:1 using Superfect (Qiagen, Courtaboeuf, France). Hygromycin B (400 μg/ml) resistant cell clones were selected, amplified, and tested for their FGF2R-DN expression by cross-linking 125I-labeled FGF-2 to cell surface FGFRs.

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1 These authors have contributed equally to this study.

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4 The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor; DN, dominant negative; CMV, cytomegalovirus; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDGF, platelet-derived growth factor.
EcoRI or EcoRV site into tetracycline-regulated pTet Splice vector (Life Technologies). C6 glioma cells were then cotransfected with pTet Splice vector containing FGFR2-DN or FGFR1-DN cDNA, the pTet-ITAK vector (Life Technologies) containing the transactivator gene, and the pCEP4 vector containing a hygromycin B-resistant gene using Superfect (Qiagen). Hygromycin-resistant cell clones were then selected with 400 μg/ml hygromycin B and 1 μg/ml tetracycline or 50 ng/ml doxycycline. Resistant clones were amplified and tested in the absence of tetracycline for their FGFR expression by cross-linking 125I-labeled FGF-2 to cell surface receptors.

**Cell Proliferation Experiments.** Proliferation assays were performed as described (8). Briefly, cells were seeded at 3000 cells on 3.5-cm diameter dishes in complete DMEM containing 7.5% FCS, 1% glutamine, and antibiotics. After overnight attachment, the cells were washed with serum-free DMEM and test medium containing 1% FCS was added. Cells were counted at specified days with a Coulter counter (Coultronics, Margency, France).

**Soft Agar Assay.** FGFR-DN-expressing cells or control cells (20,000) were put in DMEM containing 7.5% FCS and 0.2% agar (overlay) onto the top of an agar underlay (DMEM containing 7.5% FCS and 0.4% agar). Cells were fed twice a week with 1.5 ml of overlay, and the colonies (>5 cells) were counted after 2 and 3 weeks. Twenty different fields were scored from each well by two independent investigators. The experiments were done in duplicate, and the results were expressed as mean ± SD from three independent experiments.

**Binding of 125I-Labeled FGF-2 and Cross-Linking to Receptors.** FGF-2 was labeled with 125I-Na using iodogen (Pierce Corp., Rockford, IL) as coupling agent according to the manufacturer’s indications and according to Moscatelli et al. (11). The specific activity of 125I-labeled FGF-2 was 80,000–200,000 cpm/μg. FGF-2 binding experiments to high- and low-affinity sites were performed essentially as described by Moscatelli et al. (11). Cross-linking experiments of 125I-labeled FGF-2 to receptors were performed and analyzed as described by Bilkilvii et al. (8). The gels were dried and analyzed by a Phosphorimager equipped with Image Quant software (Molecular Dynamics, Sunnyvale, CA).

**Western Blotting.** Cell or tissue extracts (100 or 150 μg) were loaded onto a 15% SDS-PAGE. After electrophoresis, proteins were transferred onto a Hybond-S membrane (Amersham, Les Ulis, France). Then membranes were incubated with primary antibodies (polyclonal rabbit antihuman FGF-2 AB; Santa Cruz Biotechnology, Santa Cruz, CA), washed four times, and incubated with secondary antirabbit or goat antibodies coupled to peroxidase (Dako Corp., Trappes, France). The blots were visualized using ECL (Amersham).

**Xenografting of Tumor Cells in Immunodeficient Mice.** Transfected or control cells (500,000) were injected in DMEM s.c. in RAG 2/γc mice. Tumor growth was monitored over 27 days. Mice xenografts with Tet FGFR-DN cells were fed every day with 2 mg/ml doxycycline in 5% sucrose (Tet). The results were expressed as the maximal tumor size (as the maximum transverse diameter) ± standard deviation (SD) from three independent experiments.

**Rabbit Corneal Assay for Angiogenesis.** Five μl of C6 cell suspension were injected into the cornea of New Zealand albino rabbit eyes using a 10-μl Hamilton syringe. The injection was done 2 mm away from the limbal margin of the cornea. The potency of angiogenic activity was evaluated after 2 weeks as length of vessel extension (in mm) centrally from the limbus and as sectorial circumferential (in clock hours) involvement (13). Ten rabbit eyes were analyzed for each condition.

**RESULTS**

**Overexpression of FGFR2-DN or FGFR1-DN in Rat Glioma Cells.** To investigate the role of FGF/FGFR activity in tumor growth and angiogenesis, we transfected rat glioma C6 cells with expression vectors containing cDNAs encoding FGFR2-DN or FGFR1-DN receptors. Two expression systems were used. In the first case, we used a CMV promoter-driven expression vector (pRK5) cotransfected with a vector encoding a hygromycin-resistant gene (pCEP4). In the second case, we used a regulated system under the control of tetracycline or doxycycline. In this system, FGFR-DN expression is activated in the absence of tetracycline or doxycycline and turned off in the presence of 1 μg/ml tetracycline or 50 ng/ml doxycycline (tetoff system). A number of clones were isolated and analyzed for expression of FGFR2-DN or FGFR1-DN. Cells expressing FGFR2-DN under the control of the CMV promoter are designated as CMV FGFR2-DN cells. Cells transfected with the tetracycline-regulated system are designated as Tet+ FGFR2-DN or R1-DN cells when exposed to tetracycline or doxycycline and as Tet− FGFR2-DN or R1-DN cells in the absence of tetracycline or doxycycline. The results from two clones are shown for CMV FGFR2-DN cells (clones 18 and 2A7) and from two clones for Tet FGFR2-DN (clone 5A7 and 5B1) and Tet FGFR1-DN (clones 4A8 and 4A11).

Fig. 1 shows the expression of FGFR2-DN or FGFR1-DN of a number of clones examined. Cross-linking of 125I-labeled FGF-2 to
FGF-receptors (Fig. 1) revealed a classical pattern consisting of a band of Mr 90,000 for FGFR2-DN, Mr 60,000 for FGFR1-DN, and higher molecular weight bands corresponding to multimeric complexes. CMV or tetracycline-regulated cell clones showed different degrees of FGFR2-DN or FGFR1-DN expression. No “leakiness” in FGFR2-DN or FGFR1-DN expression was detected in Tet FR2-DN or Tet FR1-DN cells in cross-linking experiments.

Effect of FGFR-DN Expression on Tumor Cell Phenotypes in Vitro. We next investigated the effects of inhibition of FGF/FGFR activity on the cell phenotype. We performed proliferation experiments with the different cell clones as indicated in “Materials and Methods.” CMV FGFR2-DN cells grew significantly slower than untransfected control cells (Fig. 2A). At day 8, the following values of inhibition (mean \( \pm \) SD) were observed: 77.9\% \( \pm \) 0.03 (clone 18); 74.8\% \( \pm \) 0.13 (clone 2A7). Similarly, Tet FGFR2-DN or Tet FGFR1-DN were also growth inhibited in vitro when compared with Tet FGFR2-DN or Tet FGFR1-DN (Fig. 2, C and D). At day 8, the following values of inhibition (mean \( \pm \) SD) were observed for Tet cells: 64.3\% \( \pm \) 0.09 (Tet FGFR2-DN; clone 5A7), 56.5\% \( \pm \) 3.8 (Tet FGFR2-DN; clone 5B1), 34.2\% \( \pm \) 3.4 (Tet FGFR1-DN; clone 4A8), and 47.9\% \( \pm \) 0.7 (Tet FGFR1-DN; clone 4A11). Empty pTet Splice vector transfected cells grew similarly in the presence and absence of doxycycline (Fig. 2B). These results indicate that cell proliferation is inhibited in FGFR-DN-expressing C6 glioma cells.

Furthermore, anchorage-independent growth was inhibited in CMV FGFR2-DN, Tet FGFR2-DN or Tet FGFR1-DN cells (Fig. 3). The following values (mean \( \pm \) SD) of inhibition in comparison to control were observed in the different clones: 19.8\% \( \pm \) 0.4 (CMV FGFR2-DN; clone 3B8), 26.7\% \( \pm \) 1 (CMV FGFR2-DN; clone 2A7), 58.7\% \( \pm \) 3.3 (CMV FGFR2-DN; clone 18), 98.4\% \( \pm \) 1.2 (Tet FGFR2-DN; clone 5A7), 96.7\% \( \pm \) 0.6 (Tet FGFR2-DN; clone 5B1), 64.1\% \( \pm \) 7.7 (Tet FGFR1-DN; clone 4A8), and 60.6\% \( \pm \) 9.6 (Tet FGFR1-DN; clone 4A11).
Effect of the Inhibition of FGF/FGFR Activity in Mice Injected with Tumor Cells. We then investigated the growth of the different cell clones in vivo in mice (Fig. 4). The different clones were xenografted into RAG 2/γc mice, and tumor growth was measured for 27 days. RAG 2/γc mice are an allogeneic mouse strain lacking T, B, and natural killer cells (14). Initial experiments have shown that tumor take and growth were similar in nude and RAG 2/γc mice (data not shown). Control cells transfected with the hygromycin-resistant gene (Hygro cells) alone or Tet− FGF2-RDN or R1-DN cells grew actively when xenografted in immunodeficient mice.

CMV FGF2-RDN cells grew much slower than Hygro cells and were growth inhibited by 60–80% (Fig. 4A and Table 1). Furthermore, Tet− FGF2-RDN (clone 5A7) and Tet− FGF1-RDN (clone 4A11) are also strongly inhibited in comparison with control (60–80% of inhibition; Fig. 4, C and D). The stronger inhibitory effect in vivo observed for tumors derived from FGF2-RDN-expressing cells in comparison with tumors derived from FGF1-RDN cells correlated with higher FGF-RDN expression levels observed in vitro. At day 27, tumor size was significantly smaller in tumors derived from FGF2-RDN-expressing cells in comparison with control (Fig. 5; Table 1). In addition, tumor weight was decreased in CMV FGF2-RDN, Tet− FGF2-RDN or Tet− FGF1-RDN by 50–77% in comparison with control (Table 1).

Quantification of FGFR expression in tumors by Northern blotting showed that FGF2-RDN and R1-DN mRNA were expressed in all of the tumors derived from FGF2-RDN-expressing cells (Fig. 6). Furthermore, FGF-RDN mRNA expression was regulated in tumors derived from Tet FGFR-DN-expressing cells when mice were fed with doxycycline. In addition, only endogenous FGFR1 but not FGFR2 is present in tumors derived from FGF2-RDN-expressing cells, as shown for human gliomas (7).

**Fig. 4. Growth of tumors derived from s.c.-implanted, FGF2-RDN-expressing cells or control cells in immunodeficient mice.** Cells expressing FGF2-RDN or control cells were implanted s.c. in RAG 2/γc mice, and tumor size was measured twice a week for 4 weeks as indicated in “Materials and Methods.” **A,** tumors derived from CMV FGF2-RDN cells (●, clone 3B8; □, clone 2A7; ■, clone 18) or control cells (●, clone BH2). **B,** tumors derived from empty pTet Splice vector-transfected control cells, clone CA9 (●, with doxycycline; ■, without doxycycline). **C,** tumors derived from Tet FGF2-RDN cells, clone 5A7 (●, with doxycycline; ■, without doxycycline). **D,** tumors derived from Tet FGF1-RDN cells, clone 4A11 (●, with doxycycline; ■, without doxycycline). Significant difference was based on Student’s t test at days 24 and 27 for CMV FGF2-RDN-expressing tumors and at days 22, 26, and 27 for Tet FGF2-RDN-expressing tumors. **,** P < 0.01; *, P < 0.05 versus control (**) for clone 18 at day 27.

**Table 1 Reduction of volume and weight of tumors derived from FGF2-RDN-expressing cells**

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<tr>
<th>Clone</th>
<th>Volume</th>
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<tr>
<td>CMV FGF2-RDN</td>
<td>18</td>
<td>74% ± 10.1</td>
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<tr>
<td>2A7</td>
<td>71.6% ± 7</td>
<td>59.8% ± 19</td>
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<tr>
<td>3B8</td>
<td>61.6% ± 21.4</td>
<td>72% ± 12.3</td>
</tr>
<tr>
<td>Tet− FGF2-RDN</td>
<td>5A7</td>
<td>79% ± 2</td>
</tr>
<tr>
<td>Tet− FGF1-RDN</td>
<td>4A11</td>
<td>65% ± 31</td>
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**Fig. 3. Effect of FGF2-RDN or FGF1-DN expression on growth in soft agar of C6 cells.** Colonies (>5 cells) were counted in 20 different fields by two different investigators. The results are expressed as means from experiments done in duplicates; bars, SD. **A,** CMV FGF2-RDN-expressing cells (clones 3B8, 2A7, and 18) or control cells (clone BH3). **B,** Tet FGF2-RDN (clones 5A7 and 5B1), Tet FGF1-RDN cells (clones 4A8 and 4A11), or empty pTet Splice vector-transfected cells (clone CA8). □, without doxycycline; ■, with doxycycline. Significant difference was based on Student’s t test. **,** P < 0.01; *, P < 0.05 versus control.

**Effect of Inhibition of FGF/FGFR Activity after Intracerebral Transplantation of FGF2-RDN-Expressing Cells.** We next evaluated tumors generated after intracranial transplantation of transfected cells in Sprague Dawley rats (Fig. 7 and Table 2). In the control groups (rats with control C6 glialoma cell implantations), the longitudinal extension of macroscopic alterations in the right brain hemisphere (column 2 of Table 2) ranged from 3 to 5 mm. In three animals, these affected areas comprised the whole extension of the caudate putamen nucleus and an important part of the thalamic nuclei (controls 1, 2, and 3).

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*Fig. 4. Growth of tumors derived from s.c.-implanted, FGF2-RDN-expressing cells or control cells in immunodeficient mice. Cells expressing FGF2-RDN or control cells were implanted s.c. in RAG 2/γc mice, and tumor size was measured twice a week for 4 weeks as indicated in “Materials and Methods.” A, tumors derived from CMV FGF2-RDN cells (●, clone 3B8; □, clone 2A7; ■, clone 18) or control cells (●, clone BH2). B, tumors derived from empty pTet Splice vector-transfected control cells, clone CA9 (●, with doxycycline; ■, without doxycycline). C, tumors derived from Tet FGF2-RDN cells, clone 5A7 (●, with doxycycline; ■, without doxycycline). D, tumors derived from Tet FGF1-RDN cells, clone 4A11 (●, with doxycycline; ■, without doxycycline). Significant difference was based on Student’s t test at days 24 and 27 for CMV FGF2-RDN-expressing tumors and at days 22, 26, and 27 for Tet FGF2-RDN-expressing tumors. **,** P < 0.01; *, P < 0.05 versus control (**) for clone 18 at day 27.

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**Fig. 3. Effect of FGF2-RDN or FGF1-DN expression on growth in soft agar of C6 cells.** Colonies (>5 cells) were counted in 20 different fields by two different investigators. The results are expressed as means from experiments done in duplicates; bars, SD. A, CMV FGF2-RDN-expressing cells (clones 3B8, 2A7, and 18) or control cells (clone BH3). B, Tet FGF2-RDN (clones 5A7 and 5B1), Tet FGF1-RDN cells (clones 4A8 and 4A11), or empty pTet Splice vector-transfected cells (clone CA8). □, without doxycycline; ■, with doxycycline. Significant difference was based on Student’s t test. **,** P < 0.01; *, P < 0.05 versus control.
3), and they displaced other ipsilateral and contralateral brain structures (Fig. 7A). In another rat (control 4), only the caudate-putamen was affected, and the thalamus was spared. In rats implanted with clone 18 and clone 2A7, the longitudinal extension of macroscopic alterations was significantly smaller than in the control group, ranging from 1 to 2.5 mm and 1 to 1.5, respectively (Fig. 7B and C; Table 2). One of the animals implanted with clone 2A7 was discarded, because the brain showed a tumor formation on the parietal cortex, because of glioma cell spreading after implantation. Another animal of the same group did not show any morphological alteration in the right brain hemisphere. In most animals, only some areas of the caudate-putamen and/or thalamic nuclei were affected, and contralateral brain structures were less displaced than in the control group.

After histological staining of coronal brain sections, we measured the maximal transversal surface of these alterations (column 3 of Table 2), which varied between 9.5 and 23.75 mm² in the control group. However, in rats implanted with clone 18 and clone 2A7, maximal transversal surfaces were in all cases notably smaller than in control groups, varying between 3 and 8 mm² in rats implanted with clone 18 and from 0 to 5.9 mm² in rats implanted with clone 2A7. Hemorrhagic areas were not macroscopically evident in tumors developing after implantations of clones 18 and 2A7, except for small foci seen in two animals. As shown in column 4 of Table 2, the affected brain regions were completely replaced by glioma cells in two control animals. In the other two control rats, glioma cells were only detected in the periphery of tumors surrounding wide areas with necrotic brain tissue (controls 2 and 4). One brain showed several large tumor-associated hemorrhagic areas, whereas another tumor had relatively small hemorrhagic foci.

**Effect of the Expression of FGFR-DN on the Angiogenic Response.** We first analyzed vessel distribution and density by immunohistochemistry in mice with tumors from control, CMV FGFR2-DN, Tet-1 FGFR2-DN or Tet-1 FGFR1-DN cells using anti-CD31 antibodies (Fig. 8). In tumors derived from s.c. implanted control, Tet+ FGFR2-DN or Tet+ FGFR1-DN cells in RAG 2/γc mice, many vessels of different sizes were visible at the tumor margins. In contrast, tumors derived from CMV FGFR2-DN, Tet− FGFR2-DN or
Tet-2 FGFR1-DN cells xenografted into immunodeficient mice exhibited much fewer blood vessels than control tumors. Quantification of CD31-positive cells demonstrated an inhibition of 34% ± 3.8 (clone 18) and 41% ± 3.2 (clone 2A7) for tumors derived from CMV FGFR2-DN cells (Fig. 8A). Furthermore, tumors derived from Tet-FGFR2-DN (clone 5A7) or FGFR1-DN (clone 4A11) demonstrated an inhibition of 83% ± 3.4 and 41% ± 3.5, respectively (Fig. 8, C and D). No differences in vessel density was observed for control Tet clones in the presence or absence of doxycycline (Fig. 8B). Vessel density was also analyzed in intracranially implanted tumors using anti-von Willebrand antibody staining instead of anti-CD31 because of high background staining. In this case, labeling was also significantly decreased with inhibition values of 69.4% ± 10.9 (CMV FGFR2-DN; clone 18) and 67.8% ± 6.6 (CMV FGFR2-DN; clone 2A7; Fig. 8E).

Cells expressing FGFR2-DN or control cells were also implanted into the rabbit cornea. Control cells induced a strong angiogenic response (Fig. 9, A and C). Cells expressing FGFR2-DN (clone 2A7) showed a marked decrease in the induction of blood vessel growth (Fig. 9, B and C).

Effect of FGFR-DN on the Expression of VEGF, FGF-2, or FGF-4 in Tumor Cells. To get insight into the potential factors down-regulated in tumors derived from FGFR-DN expressing cells, we analyzed VEGF, FGF-2, or FGF-4 transcripts or proteins. In
DISCUSSION

To identify roles for FGF/FGFR activity in glial tumor development, we took advantage of the DN receptor strategy. Tyrosine kinase domain-deleted FGFR-DNs dimerize with endogenous receptors and thus inhibit FGF signaling (15). To ascertain that the phenotypes we observed are not related to clonal variation but to the expression of the FGFR-DNs, we used both constitutive and tetracycline-regulated expression vector systems. The tetracycline-regulated expression system has been demonstrated to be useful for in vitro and in vivo control of gene expression (16). A number of cell clones with constitutive and tetracycline-regulated expression were isolated that exhibited differences in angiogenic response and strongly support the involvement of FGF ligands such as FGF-2 or FGF-4 in tumor angiogenesis. The effect of FGF on tumor angiogenesis seems to be, at least partially, indirect and involves VEGF, thus inhibit FGF signaling (15). To ascertain that the phenotypes we observed for VEGF mRNA expression of 47 and 64.2% were observed (Fig. 10B). In contrast, FGF-2 and FGF-4 expression was not modulated in tumors derived from FGFR-DN-expressing cells when expression was analyzed by Northern or Western blotting (data not shown).

Taken together, these results indicate that cells expressing FGFR-DN exhibit a significant decrease in their angiogenic response and strongly support the involvement of FGF ligands such as FGF-2 or FGF-4 in tumor angiogenesis. The effect of FGF on tumor angiogenesis seems to be, at least partially, indirect and involves VEGF, because inhibition of FGF activity in tumors derived from FGFR-DN-expressing cells down-regulates VEGF expression in vivo.

Fig. 8. Effect of FGFR-DN expression in C6 cells on the angiogenic phenotype in tumors: quantification of vessel density. Tumors derived from s.c.-injected CMV FGFR2-DN, Tet FGFR2-DN, Tet FGFR1-DN, or control cells (A–D) in RAG 2/γ− mice were analyzed by immunohistochemistry using anti-CD31 antibodies. A, tumors derived from CMV FGFR2-DN cells (clone 2A7 and clone 18) or control (clone BH2). B, tumor derived from Tet control cells, clone CA9, with or without doxycycline. C, tumors derived from Tet FGFR2-DN cells, clone 5A7 with or without doxycycline. D, tumors derived from Tet FGFR1-DN, clone 4A11, with or without doxycycline. Five fields were analyzed for each cell clone. ×400. E, immunostaining of von Willebrand factor in intracerebral tumors derived from FGFR2-DN-expressing cells or control cells. Control (clone BH2); tumors derived from CMV FGFR2-DN cells (clone 18; clone 2A7) are shown. Five fields were analyzed for each cell clone. ×400. The data are presented as means; bars, SD. Significant difference was based on Student’s t test. **, P < 0.01; *, P < 0.05 versus control.

tumors derived from CMV FGFR2-DN cells, VEGF mRNA expression was reduced by 52–65% (Fig. 10A). In tumors derived from Tet– FGFR2-DN cells or Tet– FGFR1-DN cells, inhibition values for VEGF mRNA expression of 47 and 64.2% were observed (Fig. 10B). In contrast, FGF-2 and FGF-4 expression was not modulated in tumors derived from FGFR-DN-expressing cells when expression was analyzed by Northern or Western blotting (data not shown).

Table 2

<table>
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<td>1.5</td>
<td>0.8</td>
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** Complete (+ + +), moderate (+ +), and scarce (+) glioma replacement of altered brain regions as visualized in histological cross-sections.

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this is not attributable to clonal variation but to a true DN effect. Inhibition was stronger to some extent for FGFR2-DN-expressing cells than for FGFR1-DN-expressing cells. This is most certainly because of higher FGFR-DN expression levels achieved in FGFR2-DN-expressing cells as shown in vitro. The angiogenic response was found to be significantly inhibited in FGFR-DN-expressing cells. To explain our results, different possibilities may be considered: (a) the expression of FGF-2 or of another FGF family member is down-regulated in cells expressing FGFR-DN; (b) FGFR-DN may inhibit FGF-2 release into the extracellular compartment; (c) FGFR-DN may immobilize FGF at the cell surface, thereby sequestering extracellular FGF; (d) FGFR-DN could inhibit expression of another angiogenic factor, such as VEGF. We herein demonstrate for the first time that inhibition of endogenous FGF/FGFR activity leads to down-regulation of VEGF expression in tumors. This strongly supports the contention that FGF/FGFRs are inducing angiogenesis in tumors by an indirect mechanism that is mediated via VEGF. This is in agreement with the observations of Seghezzi et al. (22), who reported that exogenous FGF-2 induces VEGF expression in endothelial cells. In addition, FGFR-DN may not only impede the activity of angiogenic factors at a transcriptional level but may instead inhibit the mobilization of angiogenic growth factors from the tumor cell or extracellular matrix to the endothelium by inhibiting the expression or activation of proteases such as matrix metalloproteinases or plasminogen activators. This possibility is currently being investigated. Inhibition of FGF activity in human glioblastomas also down-regulates VEGF expression and inhibits tumor growth. This indicates that our results have a more general significance and also apply to human tumors. In the light of the results described herein, endogenous FGF may be part of the angiogenic switch in glial tumors, allowing tumors to progress from dormancy to invasiveness.

The implication of FGF/FGFR activity in angiogenesis has been a matter of debate. Knocking-out the gene for FGF family members, such as FGF-1, FGF-2, or FGF-8, did not reveal defects in embryonic vascular development (23–25). Furthermore, disruption of some of the FGFR family members is early lethal (26). This does not allow the analysis of the embryonic vasculature. We have recently generated transgenic mice with targeted expression of FGFR1-DN in the retinal pigmented epithelium (27). These mice display a severe defect in the development of choroidal blood vessels. This observation strongly indicates that FGF ligands are important in vascular developmental processes. The present data support an involvement of FGF in the angiogenic response in tumors. This is further supported by Fuldham et al. (28), who recently demonstrated an impairment in the angiogenic response in a FGF-2 transgenic mice. Taken together, these observations indicate that FGF may play a significant role in both developmental and repair-associated angiogenic processes.

The C6 rat glioma cells that we used as a model in our studies express a number of growth factor or growth factor receptors other than FGF ligands or FGFRs. These include VEGF and VEGFRs.

\(^5\) Unpublished results.
(29–31), interleukin 6 (32, 33), tumor necrosis factor-α (32), glial cell line-derived neurotrophic factor (34), epidermal growth factor receptors (35), or PDGF receptors (36). Overexpression of VEGF in C6 glioma cells has shown that this factor is critical for the development and maintenance of the tumor vasculature. Benjamin and Keshet (37) have used the tetracycline-regulated expression system to show that VEGF has an important role as a survival factor for tumor vessels in vivo. Blocking experiments have contributed to understand the involvement of different receptors or ligands in tumor growth. Plate et al. (29) and Millauer et al. (30) have shown that tumor growth in nude mice xenografted with C6 glioma cells was markedly inhibited when VEGFR2-DN was retrovirally targeted into animals. Furthermore, VEGFR2-DN directly expressed on tumor cells was able to inhibit tumor growth and angiogenesis (38). The inhibitory effect of VEGFR2-DN on tumor angiogenesis may be explained by adsorption of extracellular ligands. Strawn et al. (36) introduced a truncated PDGF-β receptor into C6 rat glioma cells and showed that PDGF-BB-induced tyrosine phosphorylation of the endogenous receptor was significantly reduced. In addition, these cells were growth inhibited in vitro and grew slower when xenografted in nude mice. This indicates that multiple growth factors are critical for glioma growth that may act directly on tumor cells and/or at the level of the tumor environment.

In summary, our data clearly demonstrate that FGF/FGFR activity is involved in glioma growth in vitro and in vivo. A therapeutic strategy based on the inhibition of FGFR function may be useful for the treatment of solid FGF-producing tumors, such as gliomas, and may disrupt both angiogenesis-dependent and -independent signaling. This is similar to the effect of maspin, a recently discovered angiogenesis inhibitor, which also directly impedes tumor growth in addition to its effect on angiogenesis (39). Thus, inhibition of the FGF signal transduction pathway may constitute an interesting target for therapeutic intervention.

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Inhibition of Fibroblast Growth Factor/Fibroblast Growth Factor Receptor Activity in Glioma Cells Impedes Tumor Growth by Both Angiogenesis-dependent and -independent Mechanisms

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