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

Patrick Auguste, Demirkan B. Gürsel, Sylvie Lemière, Diana Reimers, Pedro Cuevas, Fernando Carceller, James P. Di Santo, and Andreas Bikfalvi

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 FGF2 (FGFR2-DN) or FGFR1 (FGFR1-DN) in glioma C6 cells by using constitutive or tetracycline-regulated expression systems. Anchor-age-dependent or independent growth was inhibited in FGFR-DN-expressing cells. Tumor development after xenografting FGFR-DN-expressing cells in immunodeficient mice or after transplantation in rat brain was strongly inhibited. Quantification of microvessels demonstrated a significant decrease in vessel density in tumors derived from FGFR-DN-expressing cells. Furthermore, in a rabbit corneal assay, the angiogenic response after implantation of FGFR-DN-expressing cells was decreased. In tumors expressing FGFR-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 (1, 2). 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 IIc variants, whereas FGF-7 binds to IIb 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) or 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 FGFR1 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- (2 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 FGFR-DN strategy (8–10). Furthermore, we used a tetracycline-regulated expression system to control FGFR2-DN and FGFR1-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 FGFR2-DN in C6 glioma cells, C6 glioma cells were cotransfected with the pRK5 expression vector containing human FGFR2-DN cDNA (tyrosine kinase domain deleted, 3 immunoglobulin-like loop form, IIc splicing 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 1:10 using Superfect (Qiagen, University Medical Center, New York, NY) and the pCEP4 vector containing a hygromycin-resistant gene at a ratio of 1:10 using Superfect (Qiagen, Courtaboeuf, France). Hygromycin B (400 µg/ml) resistant cell clones were selected, amplified, and tested for their FGFR2-DN expression by cross-linking 3H-labeled FGF-2 to cell surface FGFRs.

For the tetracycline-regulated expression system, human FGFR2-DN or mouse FGFR1-DN (tyrosine kinase domain deleted, 2 immunoglobulin-like loop form, IIc splice variant; kindly provided by Dr. L. Williams, University of California San Francisco, San Francisco, CA) cDNA were cloned at the

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These authors have contributed equally to this study.

To whom requests for reprints should be addressed, at Growth Factor and Cell Differentiation Laboratory, University Bordeaux I, Avenue des Facultés, 33 405 Talence, France.

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.

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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. A 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 specificity of 125I-labeled FGF-2 was 80,000–200,000 cpm/ng. 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 Biktalvi 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 anti-human FGF-2 AB; polyclonal goat anti-human FGF-4 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 + clones) or with 5% sucrose solution alone (Tet − clones). Mice xenografted with CMV FGFR2-DN or hygromycin-resistant control cells were fed with water alone. Ten animals were analyzed for each clone. Tumor measurements were made in two directions using calipers, and tumor volume was calculated by using a2 × b/2, where a is the width and b the length of the tumor. At the end of the experiment, animals were sacrificed, and tumor weight was determined. Tumor tissue was then processed for histology or immunohistology (see below).

Northern Blot Analysis. RNA (30 μg) extracted from tumors derived from FGFR-DN or control cells was run on a 1% formaldehyde-agarose gel and transferred to a positively charged nylon membrane (Amersham Life Technologies). After baking the membrane for 2 h at 80°C, the membrane was prehybridized in a solution containing 50% formamide, 5× SSPE, 5× Denhardt’s solution, 0.5% SDS and denatured salmon sperm DNA (100 ng/ml) for 4 h at 42°C. Hybridization was done overnight at 42°C with Megaprime random-labeled (32P-ATP) probes (Amersham). The following probes were used: 1100-bp truncated mouse FGFR1 or 1300-bp human FGFR2 probes, 576-bp human VEGF165 full-length probe, or a full-length 1300-bp rat GAPDH probe. After hybridization, membranes were washed twice in 1× SSPE, 0.1% SDS at 42°C for 20 min. For high stringency, a third wash was performed twice in 0.1× SSPE, 0.1% SDS at 55°C for 10 min. The results were analyzed with a PhosphorImager and Image Quant software.

Implantation of Tumor Cells into the Rat Brain. A midline skin incision was made, and a small burr hole was drilled in the skull 3 mm lateral to the bregma in anesthetized male Sprague Dawley rats (250–300 g). Animals were placed into a stereotactic frame, and 2 μl of cell suspension, containing 100,000 C6 glioma cells, were injected into the right caudate-putamen placed at the following stereotactic coordinates from bregma (nose bar at +5): ML, 3 mm; AP−, 0.2 mm; and DV, 5 mm. Groups of four rats were used for each type of C6 glioma clone. Thirty days after surgery, animals were used intraocular perfusion with 4% paraformaldehyde, and the whole brains were dissected and photographed. Serial 1-mm transversal sections were cut, and tumor longitudinal extension was measured by adding serial sections where macroscopic alterations could be detected under a surgical microscope. Selected sections with tumor alterations displaying maximal transversal extensions were processed for paraffin embedding. Subsequently, 8-μm sections were cut and processed for both immunohistochemistry and histological analysis. Stained H&E sections were used to measure tumor maximal transversal surface, and immunostained brain sections were used for the quantification of tumor angiogenesis.

Histology and Immunohistochemistry. Paraffin-embedded tumor tissue was cut into 5-μm sections, rehydrated, and processed for histology or immunohistochemistry. For labeling with anti-CD31 antibody, sections were also preincubated with 0.1% trypsin in PBS. Blocking was done for 2 h in PBS containing 0.1% Tween 20 and 1 mg/ml BSA (buffer A). Slides were then incubated with anti-CD31 antibody (MEC 13; Becton Dickinson, le Pont de Claix, France) at 1:500 dilution in buffer A. After washing, the slides were incubated with biotinylated anti-rat antibody (Dako) in buffer A. Subsequently, the slides were washed again and incubated with ABC reagent (Vectastain; Vector, Burlingame, CA) and 3,3′-diaminobenzidine (Dako). Counterstaining was done with Harris hematoxylin.

For von Willebrand factor staining, 8-μm sections were rehydrated and boiled for 10 min in 10 mM citrate buffer (pH 6) in a microwave oven. Incubation with primary antibodies (rabbit polyclonal antihuman von Willebrand factor antibody; Dako), secondary antibodies (biotinylated goat antirabbit polyclonal antibody; Vectastain), and revelation were done as described above. Vessel density was quantified as described (12).

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

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FGF-receptors (Fig. 1) revealed a classical pattern consisting of a band of \( M_r 90,000 \) for FGFR2-DN, \( M_r 60,000 \) 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+ FGFR2-DN or Tet+ FGFR1-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 ± SD) were observed: 77.9% ± 0.03 (clone 18); 74.8% ± 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 ± SD) were observed for Tet− cells: 64.3% ± 0.09 (Tet− FGFR2-DN; clone 5A7), 56.5% ± 3.8 (Tet− FGFR2-DN; clone 5B1), 34.2% ± 3.4 (Tet− FGFR1-DN; clone 4A8), and 47.9% ± 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 ± SD) of inhibition in comparison to control were observed in the different clones: 19.8% ± 0.4 (CMV FGFR2-DN; clone 3B8), 26.7% ± 1 (CMV FGFR2-DN; clone 2A7), 58.7% ± 3.3 (CMV FGFR2-DN; clone 18), 98.4% ± 1.2 (Tet− FGFR2-DN; clone 5A7), 96.7% ± 0.6 (Tet− FGFR2-DN; clone 5B1), 64.1% ± 7.7 (Tet− FGFR1-DN; clone 4A8), and 60.6% ± 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 alymphoid 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− FGFR2-DN or R1-DN cells grew actively when xenografted in immunodeficient mice.

CMV FGFR2-DN cells grew much slower than Hygro cells and were growth inhibited by 60–80% (Fig. 4A and Table 1). Furthermore, Tet− FGFR2-DN (clone 5A7) and Tet− FGFR1-DN (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 FGFR2-DN-expressing cells in comparison with tumors derived from FGFR1-DN cells correlated with higher FGFR-DN expression levels observed in vitro. At day 27, tumor size was significantly smaller in tumors derived from FGFR-DN-expressing cells in comparison with control (Fig. 5; Table 1). In addition, tumor weight was decreased in CMV FGFR2-DN, Tet− FGFR2-DN or Tet− FGFR1-DN by 50–77% in comparison with control (Table 1).

Quantification of FGFR expression in tumors by Northern blotting showed that FGFR2-DN and R1-DN mRNA were expressed in all of the tumors derived from FGFR-DN-expressing cells (Fig. 6). Furthermore, FGFR-DN 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 FGFR-DN-expressing cells, as shown for human gliomas (7).

**Effect of Inhibition of FGF/FGFR Activity after Intracerebral Transplantation of FGFR-DN-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 glioma 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).

![Image](cancersres.aacrjournals.org)
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. 7, B 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 FGFR2-DN or Tet 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– 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
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).

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.

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 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 different FGFR2-DN or FGFR1-DN expression levels. In tumors derived from FGFR-DN-expressing cells, inhibition of FGF activity in tumors derived from FGFR-DN-expressing cells down-regulates VEGF expression in vivo.

Table 2

Quantitative analysis of tumors derived from FGFR2-DN cells transplanted in the rat brain

<table>
<thead>
<tr>
<th>Rats</th>
<th>Longitudinal extension (mm)</th>
<th>Maximal transversal surface (mm²)</th>
<th>Replacement by glioma cells*</th>
</tr>
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<tbody>
<tr>
<td>Control 1</td>
<td>3.5</td>
<td>20</td>
<td>++</td>
</tr>
<tr>
<td>Control 2</td>
<td>5</td>
<td>21</td>
<td>++</td>
</tr>
<tr>
<td>Control 3</td>
<td>5</td>
<td>23.75</td>
<td>++</td>
</tr>
<tr>
<td>Control 4</td>
<td>3</td>
<td>9.5</td>
<td>++</td>
</tr>
<tr>
<td>Clone 18, rat 1</td>
<td>2.5</td>
<td>7</td>
<td>++</td>
</tr>
<tr>
<td>Clone 18, rat 2</td>
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<td>++</td>
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<tr>
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<td>1</td>
<td>5.9</td>
<td>++</td>
</tr>
<tr>
<td>Clone 2A7, rat 3</td>
<td>1.5</td>
<td>0.8</td>
<td>++</td>
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</table>

* Complete (+++), moderate (++), and scarce (+) glioma replacement of altered brain regions as visualized in histological cross-sections.

In vivo growth was strongly impaired when cells with constitutive or tetracycline-regulated FGFR2-DN or FGFR1-DN expression were xenografted in immunodeficient mice. This demonstrates that FGFs are important for glioma development in vivo. This is in agreement with Yayon et al. (21), who also demonstrated the participation of autocrine FGF in melanoma cell growth. The strong inhibition in vivo observed for cells expressing FGFR2-DN or FGFR1-DN under the control of tetracycline or doxycycline clearly demonstrates again that cell types used in these studies (transformed cells in this study and immortalized cells in the previous study).

Anchorage-independent growth was inhibited in cells expressing FGFR2-DN or FGFR1-DN. This indicates that some of the properties related to cell transformation are inhibited by the expression of FGFR-DN in tumor cells. This may include effects on integrins or protease production that are involved in interactions with the extracellular matrix. This suggests that signaling through FGFRs modulates adhesive interactions with the extracellular matrix. A number of observations are in support of this contention. FGF-2 modulates integrin expression and adhesion to the extracellular matrix in endothelial cells (18, 19). Furthermore, endogenous M* 18,000 FGF-2 has been demonstrated to interfere with the expression and function of αβ1 and α5β1 integrins in NIH 3T3 cells (20).

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Anchorage-independent growth was inhibited in cells expressing FGFR2-DN or FGFR1-DN. This indicates that some of the properties related to cell transformation are inhibited by the expression of FGFR-DN in tumor cells. This may include effects on integrins or protease production that are involved in interactions with the extracellular matrix. This suggests that signaling through FGFRs modulates adhesive interactions with the extracellular matrix. A number of observations are in support of this contention. FGF-2 modulates integrin expression and adhesion to the extracellular matrix in endothelial cells (18, 19). Furthermore, endogenous M* 18,000 FGF-2 has been demonstrated to interfere with the expression and function of αβ1 and α5β1 integrins in NIH 3T3 cells (20).

In vivo growth was strongly impaired when cells with constitutive or tetracycline-regulated FGFR2-DN or FGFR1-DN expression were xenografted in immunodeficient mice. This demonstrates that FGFs are important for glioma development in vivo. This is in agreement with Yayon et al. (21), who also demonstrated the participation of autocrine FGF in melanoma cell growth. The strong inhibition in vivo observed for cells expressing FGFR2-DN or FGFR1-DN under the control of tetracycline or doxycycline clearly demonstrates again that...
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 unimpairment 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

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Unpublished results.

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Fig. 9. Effect of FGFR2-DN expression in C6 cells on the angiogenic response in the rabbit cornea assay. Cells expressing FGFR2-DN (clone 2A7) or hygromycin-resistant control cells (clone BH3) were implanted into the rabbit cornea. Corneal neovascularization was assessed as the length of the vessel extension centrally from the limbus and the sectorial circumferential involvement. A, control (clone BH3). B, FGFR2-DN-expressing cells (clone 2A7). C, quantification of vessel density (mean values; bars, SD. Ten eyes were analyzed for each condition.
Fig. 10. Expression of VEGF in tumors derived from FGFR-DN-expressing cells or control cells. Total RNA was extracted from tumors grown in RAG 2/γc mice and analyzed as indicated in "Materials and Methods." A, CMV FGFR2-DN (clones 18, 2A7, and 3B8) or control tumors (clone BH2). B, Tet FGFR2-DN (clones 5A7 and 5B1) or Tet FGFR1-DN (clone 4A11) in the presence (+) or absence (−) of doxycycline.

(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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