A Common Mutant Epidermal Growth Factor Receptor Confers Enhanced Tumorigenicity on Human Glioblastoma Cells by Increasing Proliferation and Reducing Apoptosis

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ABSTRACT

Alterations of the EGFR gene occur frequently in human gliomas where the most common is an in-frame deletion of exons 2-7 from the extracellular domain, resulting in a truncated mutant receptor (ΔEGFR or del 2-7 EGFR). We previously demonstrated that introduction of ΔEGFR into human U87MG glioblastoma cells (U87MG.ΔEGFR) conferred remarkably enhanced tumorigenicity in vivo. Here, we show by cell-mixing experiments that the enhanced tumorigenicity conferred by ΔEGFR is attributable to a growth advantage intrinsic to cells expressing the mutant receptor. We analyzed the labeling index of the proliferation markers Ki67 and bromodeoxyuridine and found that tumors derived from U87MG.ΔEGFR cells had significantly higher labeling indexes than those of tumors derived from U87MG cells that were either naive, expressed kinase-deficient mutants of ΔEGFR, or overexpressed exogenous wild-type EGFR. We also utilized terminal deoxynucleotidyl transferase-mediated nick end-labeling assays and showed that the apoptotic index of U87MG.ΔEGFR tumors was more than 4-fold lower than that of parental U87MG tumors. This decrease in cell death was inversely correlated with the expression level of Bcl-XL, a negative regulator of apoptosis, which was more than 3-fold higher in U87MG.ΔEGFR-derived tumors than in those derived from parental cells. Similar observations were obtained in vitro in serum-free conditions. These results suggest that ΔEGFR exerts its pronounced enhancement of glioblastoma tumorigenicity by stimulating proliferation and inhibiting apoptosis and that the effects are directly attributable to its constitutively active signal.

INTRODUCTION

Gliomas are the most common primary tumor of the CNS. The most malignant type of glioma, glioblastoma multiforme, has a survival rate of less than 5% over 5 years (1). Malignant gliomas originate and progress through clonal evolution, a multistep process by which genetic alterations that are advantageous to growth accumulate, often in a defined relative order (2, 3). These genetic events include mutation of the p53 gene (4), p16/CDKN2 gene inactivation (5), amplification and rearrangement of the EGFR gene (6, 7), and monosomy of chromosome 10 (2).

Several lines of evidence have implicated abnormalities in EGFR signaling in tumorigenesis. First, the overexpression of EGFR leads to ligand-dependent transformation of immortalized rodent fibroblasts (8). Second, the v-erbB oncogene, derived from the avian erythroblastosis virus, encodes a truncated and mutated form of EGFR that has a high level of tyrosine kinase activity in the absence of ligand (9).

Finally, amplification and rearrangement of the EGFR gene is frequently observed in a variety of human malignant neoplasms, including carcinomas of the breast (10), lung (11), kidney (12), ovary (12), cervix (12), and squamous cells (13). In gliomas, EGFR gene amplification and overexpression are restricted to high-grade tumors, especially those of grade IV (glioblastoma), where they occur at a frequency of 40–50% (14–16). Nearly one half of the tumors that show amplification also show rearrangement of the gene, resulting in tumors expressing endogenous wild-type EGFR as well as the episcopal mutant forms (7, 14). The most common of the abnormalities in the EGFR in gliomas are genomic rearrangements leading to specific elimination of a DNA fragment containing exons 2-7 of the gene, which causes an in-frame deletion of 801 bp of the coding sequence in the extracellular domain of the receptor (6, 7, 17). Overexpression of this mutant to levels comparable to those seen in primary brain tumors in the human glioblastoma cell line U87MG leads to the presence of mutant receptors of 140–155-kilodaltons at the cell surface in which tyrosine residues are constitutively phosphorylated independent of EGF stimulation, whereas the endogenous receptor of 170 kilodaltons is phosphorylated only in response to ligand (18). Although the expression of the mutant EGFR does not dramatically affect the in vitro growth rate of cells cultured with serum, their tumorigenic capacity is remarkably enhanced when implanted s.c. or intracerebrally into immune-deficient mice (18). This is consistent with the observation that its presence is prognostic of a shorter interval to clinical relapse and poorer survival (15).

Genetic alterations can bestow growth advantages on tumor cells by a variety of biological mechanisms. Accumulation of genetic alterations may result in a substantial growth advantage for single cells, leading to subsequent clonal expansion (19). Some genetic events facilitate the interaction of cells with their microenvironment, thereby exerting a field effect, such as are seen in angiogenesis and paracrine growth factor stimulation, favoring the expansion of the tumor as a whole (16, 20). Glioma growth itself is determined by the balance between cell proliferation and cell death, the latter comprising the distinct processes of necrosis and apoptosis (21). In contrast to necrosis, which usually occurs as the outcome of catastrophic injury to the cell (22), apoptosis is considered a physiological regulator of cell number in normal and neoplastic tissues and can be regulated by specific genes including oncogenes (21, 22). In certain human solid malignancies, including gliomas, apoptosis has been shown to occur spontaneously, suggesting that it may have a profound effect on tumor formation and progression (23). All of these mechanisms could potentially play a role in the enhanced tumorigenicity conferred by ΔEGFR in vivo.

Here, we demonstrate that a small number of U87MG.ΔEGFR cells outgrow a large excess of the parental U87MG cells in an in vivo glioma formation model in mouse brain. This dramatic clonal expansion results in tumors composed almost entirely of ΔEGFR-expressing cells and suggests that the growth advantage bestowed by this mutant protein is intrinsic and exerted at the single-cell level. This growth advantage was due not only to an increased proliferation rate,
but also to a reduced rate of apoptosis which was inversely correlated with expression levels of Bcl-XL, a negative regulator of apoptosis (24). Furthermore, a kinase-deficient form of ΔEGFR was unable to confer similar growth advantages. Overexpression of wild-type EGFR was similarly ineffective. These phenomena also became evident in vitro when cells were deprived of serum. These data suggest that in glioblastoma cells, ΔEGFR exerts its biological effect by virtue of constitutive activation of its tyrosine kinase, which emanates signals leading to pronounce tumorigenic activity.

MATERIALS AND METHODS

Cells and Culture. The human glioma cell line U87MG was purchased from American Tissue Culture Collection (25). The generation of the U87MG.ΔEGFR cells expressing the exogenously truncated EGFR which lacks a part of the extracellular domain of the receptor has been described previously (18). U87MG.ΔEGFR cells were labeled by expression of the human placental Akp gene by infecting cells with a retrovirus carrying hygromycin resistance and Akp genes (26). Akp expression by U87MG.ΔEGFR-Akp cells was studied prior to their injection into nude mouse brain, confirming that more than 95% of the cells expressed Akp in vitro (data not shown). U87MG cells were coinfected with a retrovirus carrying Lux and neomycin resistance genes along with expression of the human placental Akp gene by infecting cells with a retrovirus carrying hygromycin resistance and Akp genes (26). Akp expression by U87MG.ΔEGFR-Akp cells was studied prior to their injection into nude mouse brain, confirming that more than 95% of the cells expressed Akp in vitro (data not shown). U87MG cells were coinfectected with a retrovirus carrying Lux and neomycin resistance genes along with a retrovirus vector harboring LacZ and hygromycin resistance genes. These U87MG.Lux-LacZ cells were used as controls for U87MG.ΔEGFR-Akp cells because they harbored a similar set of retroviral constructs and exhibited similar drug resistance, being resistant to both neomycin and hygromycin.

The kinase-deficient human EGFR mutant DK, with a lysine 721 to methionine substitution at the AlP-binding site, was obtained by site-directed mutagenesis. The retroviral vector pLRNL was used for gene transfer of these ΔEGFR mutants into U87MG cells as described previously (18). A retroviral vector encoding wild-type EGFR, as well as the hygromycin resistance gene, was used to infect U87MG cells to obtain wild-type EGFR-overexpressing cells. The steady-state expression levels of these various EGFR proteins in U87MG cells were determined by fluorescence-activated cell sorter analysis, confirming equivalent amounts of cell surface protein for all of the constructs used.

U87MG.ΔEGFR-Akp and U87MG.Lux-LacZ were maintained in medium containing 400 μg/ml G418 (Life Technologies, Inc., Grand Island, NY) and 200 μg/ml hygromycin (Boehringer Mannheim, Indianapolis, IN). U87MG.ΔEGFR and U87MG.DK were maintained in the presence of 400 μg/ml G418. U87MG.wtEGFR was maintained with 200 μg/ml hygromycin. All cells were cultured in DMEM supplemented with 10% heat-inactivated bovine serum (Hyclone, Logan, UT), 2 mm glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C.

Stereotactic Inoculation of Tumor Cells into Nude Mouse Brain. For intracerebral stereotactic inoculation, cells were resuspended in 5 μl of PBS and inoculated into the right corpus striatum of the brain of 4−5-week-old female nude mice of BALB/c background (18). Brains were removed at various time points and fixed. Tumor sizes were determined microscopically.

In experiments where cell types were mixed prior to injection, 5 × 10⁵ U87MG.Lux-LacZ cells were injected with an admixture of either 10 or 50 U87MG.ΔEGFR-Akp cells. As controls, 5 × 10⁵ cells of U87MG.Lux-LacZ alone or 400 cells of U87MG.ΔEGFR-Akp cells alone were injected. In experiments where proliferation and apoptosis were determined, between 800 and 5 × 10⁵ cells of a given cell type were injected to produce tumors of a similar size at a similar time from cells with very different growth rates.

Akp Staining in Mixed Tumor Sections. Brains were fixed by perfusion with 4% paraformaldehyde followed by further fixation in 4% paraformaldehyde after removal and then immersed in 30% sucrose in PBS at 4°C. Tissues were embedded in OCT (Miles, Elkhart, IN), frozen in liquid nitrogen, and stored at −80°C. Thin cryostat sections (5−7 μm) wereimmersed in 4% paraformaldehyde, washed in PBS containing 2% MgCl₂, and treated in PBS at 65°C. Then sections were rinsed in X-P detection buffer (100 mm Tris-HCl (pH 9.5), 100 mm NaCl, and 50 mm MgCl₂) for 10 min and incubated in a X-P reaction mix [0.1 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, 1 mg/ml nitroblue tetrazolium (Sigma, St. Louis, MO), and 0.5 mm levamisole in a X-P detection buffer] for 4 to 8 h at room temperature. The reaction was terminated by washing in PBS containing 20 mm EDTA, and the sections were mounted after counterstaining with 1 μg/ml Hoechst 33258 (Sigma). Akp-positive cells and nuclei in the tumor were identified using light and fluorescence microscopy, respectively, and the proportion of Akp-positive cells was calculated.

KI-67 Immunostaining. Brains were removed, embedded in OCT compound, frozen in liquid nitrogen, and stored at −80°C. Thin (5 μm) cryostat sections were fixed in ice-cold acetone for 20 min followed by air drying for 30 min. After equilibration, endogenous peroxidase activity was quenched with a 2% hydrogen peroxide solution in (PBS) for 30 min and rinsed in PBS for 15 min. Sections were then blocked in 10% normal goat serum (in PBS) for 10 min. Prediluted rabbit anti-human Ki-67 polyclonal antibody (DAKO, Carpinteria, CA) was then administered, and incubation was conducted for 12 h at 4°C. After rinsing in PBS, a goat anti-rabbit immunoglobulin secondary antibody (Vector, Burlingame, CA) was applied at 1:200 dilution for 30 min at room temperature. Sections were rinsed in PBS and then incubated with the avidin-biotin complex (Vector) for 30 min. A final PBS rinse then preceded application of the substrate mix consisting of 0.5 mg/ml diaminobenzidine (Sigma) 0.7 mg/ml sodium azide (Sigma), and 6.8 mg/ml imidazole (Sigma) in PBS. Sections were exposed to the substrate for 3 to 5 min in the presence of hydrogen peroxide, rinsed in PBS, counterstained in hematoxylin, and then mounted. The percentage of reactive tumor nuclei to the Ki-67 antibody was estimated following examination of 10 high-power fields. An average of 1000 cells was examined for scoring in areas of tumor judged to be most representative.

BrdUrd Incorporation in DNA Synthesis. DNA synthesis rates in vivo were evaluated with BrdUrd incorporation. BrdUrd (30 μg/kg; Amersham, Arlington Heights, IL) was administered i.p. to the nude mice 30 min before sacrifice at approximately 3−4 weeks after inoculation. Removed brains containing tumors were fixed in chilled 70% ethanol for 12 h, embedded in paraffin, and cut into sections 4-μm thick. The sections were deparaffinized, rehydrated, and incubated for 20 min in 2 N HCl to denature DNA. After washing in PBS, the sections were immunostained for BrdUrd by reaction with a prediluted mouse monoclonal antibody to BrdUrd (Amersham) for 1 h at room temperature and then a secondary rhodamine-conjugated antimouse immunoglobulin antibody (Jackson ImmunoResearch, West Grove, PA) at a 1:100 dilution for 30 min. Nuclei of the cells were stained with 2 μg/ml Hoechst 33258 for 10 min. To calculate the BrdUrd LI, more than 2000 cells were examined in each tissue section. For in vitro assays, cells were plated at 60−70% confluency on 12-mm coverslips in fully supplemented medium for 24 h. Then the cells were subjected to serum starvation for 32 h followed by labeling with BrdUrd under serum-free conditions for 24 h. The cells were fixed in 95% ethanol and 5% glacial acetic acid and subsequently immunostained for DNA synthesis as described above. To calculate BrdUrd LI, 500 cells were examined in each coverslip using fluorescence microscopy.

In Situ (TUNEL) Labeling of Apoptotic DNA Fragmentation. In paraffin-embedded, 10% formalin-fixed tissue sections of xenografted tumors in the nude mouse brain removed 3−5 weeks after inoculation, apoptotic cells were visualized using TUNEL of apoptotic DNA strand breaks (27). Tissue sections at 4-μm thickness were deparaffinized, rehydrated, and treated with 20 μg/ml proteinase K (Boehringer Mannheim). The slides were then incubated with TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and its substrate fluorescein-conjugated dUTP using an In Situ Cell Death Detection kit-fluorescein (Boehringer Mannheim) at 37°C for 1 h in the dark. Positive control sections were pretreated with 2 units/ml DNase 1 (Boehringer Mannheim) in 20 mm Tris (pH 7.0) and 5 mm MgCl₂ for 10 min at room temperature, and negative controls were incubated without terminal deoxynucleotidyl transferase. Following incubation, the sections were washed in PBS and incubated with 1 μg/ml Hoechst 33258 in 10 min at room temperature and washed in PBS. Apoptotic (FITC-labeled nuclei which were green) cells in each tissue section were visualized with fluorescence microscopy and counted by examination of 1000−4000 cells. The AI was calculated as a ratio of apoptotic cell number:total tumor cell (Hoechst-stained nuclei which were blue) number within the areas. For in vitro assays, cells plated on coverslips were starved for 75 h and subsequently fixed in 4% paraformaldehyde for 30 min. Following permeabilization in Triton X-100/sodium citrate, apoptotic cells were labeled by TUNEL reaction as described above. To calculate AI, more than 1000 cells were examined in each coverslip.
Northern Blotting. Total RNAs from frozen tumor tissues ground in liquid nitrogen and cultured cells under serum starvation for 48 h were isolated using Trizol (Life Technologies, Inc.). Fifteen μg of total RNA were electrophoresed on a 1% agarose formaldehyde gel, transferred onto a nylon filter (Hybond N*; Amersham), and visualized using methylene blue staining (28). The filter was then hybridized with 32P-labeled human bcl-Xi cDNA (gift from S. J. Korsmeyer, Washington University, St. Louis, MO) at a specific activity of 106 cpm/ml in a hybridization buffer containing 50% formamide, 1× Denhardt’s solution, 0.5% SDS, 5× SSC, 10% dextran sulfate, and 100 μg/ml herring sperm DNA at 42°C for 15 h. The filter was washed in 0.5× SSC and 0.1% SDS at 50°C for 15 min and exposed to Kodak Biomax film at ~80°C with an intensifying screen. Quantitation was carried out using laser scanning densitometry of the autoradiogram.

Western Blotting. To obtain whole tumor lysates, frozen tumor samples were ground in liquid nitrogen and lysed in sample buffer containing 0.1 M Tris-HCl (pH 6.8), 2% SDS, 3.2 M urea, 4% 2-mercaptoethanol, 0.25% bromphenol blue, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 5 μg/ml antipain, 2 mM sodium o-vanadate, and 10 mM sodium PPi. Cultured cells were lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and inhibitors of proteases and phosphatases. The lysates were boiled and centrifuged to remove cell debris prior to separation of proteins on 12.5% SDS-polyacrylamide gels and transfer to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked for 1 h at room temperature in 5% nonfat milk/TBST [0.1% Tween 20, 125 mM NaCl, 25 mM Tris (pH 7.5), and 0.1% sodium azide] and probed with anti-Bcl-X rabbit polyclonal antibody (Transduction, Lexington, KY), anti-Bcl-2 hamster monoclonal antibody (PharMingen, San Diego, CA), or anti-Bax rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. Blots were then incubated with horseradish peroxidase conjugated antibodies and visualized with enhanced chemiluminescence detection, according to the manufacturer’s instructions (Amersham). Quantitation was carried out using laser scanning densitometry of the autoradiogram.

RESULTS

Clonal Expansion of U87MG.ΔEGFR Cells. The enhancement of tumorigenesis conferred by expression of ΔEGFR (18) could be mediated at the single-cell level or could be due to a positive field effect on surrounding tumor cells or both. To distinguish among these possibilities, we mixed U87MG.ΔEGFR cells identifiable by their stable expression of the Akp gene (U87MG.ΔEGFR-Akp) with 5 × 103 U87MG cells double-infected with luciferase and LacZ gene-carrying retroviruses (U87MG.Lux-LacZ) at 1:10,000 and 1:50,000 ratios and injected them into nude mouse brains. Within 4–5 weeks after injection, U87MG.ΔEGFR-Akp cells, identified by Akp-positive staining, had outgrown the U87MG.Lux-LacZ cells dramatically, with the percentage of U87MG.ΔEGFR-Akp cells reaching 95% (20:1 ratio) and 83% (5:1 ratio) in tumors where the original ratio was 1:10,000 and 1:50,000, respectively (Fig. 1, b and c). Control tumors derived from 5 × 103 U87MG.Lux-LacZ cells formed brain tumors that were completely negative for Akp staining and grew much slower than tumors that also contained U87MG.ΔEGFR-Akp cells (Fig. 1a). Tumors formed from 400 U87MG.ΔEGFR-Akp cells developed rapidly and showed 100% positive staining for Akp. The growth rate of these tumors was similar to that of the mixed tumors initiated with 1:50,000 U87MG.Lux-LacZ cells (Fig. 1d). Similar results were obtained when U87MG cells were alternately labeled by Akp instead of U87MG.ΔEGFR cells. Since U87MG.ΔEGFR cells were originally mixed with parental U87MG cells at extremely low ratios, it is unlikely that U87MG.ΔEGFR cells promoted the growth of U87MG cells surrounding them, since this would have resulted in tumors with cell-type ratios closer to the original mixtures. Thus, these experiments suggest that ΔEGFR overexpressed in U87MG cells exerts its biological functions not by a positive field effect on all surrounding cells, but rather by bestowing a cell intrinsic growth advantage.

Increased Proliferation in U87MG.ΔEGFR Cells in Vivo and in Vitro under Serum Starvation. We next tested whether this intrinsic growth advantage conferred by ΔEGFR resulted from an elevated proliferation rate, reduced apoptosis rate, or both. To evaluate the proliferation rate of tumor cells, Ki-67 immunostaining was applied to frozen sections of xenografted brain tumors. Ki-67 is a proliferation-associated nuclear antigen present throughout the cell cycle, and the Ki-67 LI provides a reliable means for evaluating the growth fraction of a tumor cell population (29). The Ki-67 LI of U87MG, U87MG.ΔEGFR, U87MG.DK, and U87MG.wtEGFR brain tumors was compared in two different settings: tumors that had developed after a short incubation period (12 days after implantation) and tumors that had grown to a large size in longer incubation times (more than 22 days). The results showed that U87MG.ΔEGFR tumors had a significantly higher Ki-67 LI than parental U87MG tumors (Ki-67 LI of 58% and ~20%, respectively) regardless of the incubation time (P < 0.001, Student’s t test). Similarly, Ki-67 LIs of tumor cells overexpressing the kinase-deficient form of ΔEGFR or wild-type EGFR were also lower than that of U87MG.ΔEGFR tumors in both short (up to 16%) (P < 0.001) and long incubation periods (up to 29%, P < 0.01; Fig. 2A).

We further investigated BrdUrd LI in these tumors as a second and independent parameter of proliferation rate, it being a direct measurement of the percentage of cells undergoing DNA synthesis at a given
time (30). The moderate-sized tumors which had grown for 3 to 4 weeks following implantation of U87MG.ΔEGFR cells had a BrdUrd LI of 28.6%, which was significantly higher than that of U87MG-derived tumors (16.0%, P < 0.01) and of any of the other types of tumors (P < 0.01; Fig. 2B). The BrdUrd LI of U87MG.ΔEGFR tumors indicates a strikingly high proliferation rate when compared to that of glioblastomas in clinical specimens which average 7.3% (31). These differences in proliferation rate could be also observed in vitro when cells were cultured in serum-free medium. As shown in Fig. 2C, U87MG.ΔEGFR cells retained a significantly higher BrdUrd LI (36.4%) than parental U87MG cells (14.0%, P < 0.001) even after a 56-h serum starvation. The kinase-deficient mutant of ΔEGFR mostly lost this enhanced proliferative activity (P < 0.001), although a small increase in the BrdUrd LI was observed in U87MG.DK cells compared to that in U87MG cells. Overexpression of wild-type EGFR also showed a significantly lower effect than ΔEGFR (P < 0.001) under starvation conditions. Acute stimulation of overexpressed wild-type EGFR by addition of EGF to the serum-free medium, however, effectively increased the proliferation rate of U87MG.wtEGFR cells (Fig. 2C). These BrdUrd LI data were consistent with the observed Ki-67 LI in vivo, strongly suggesting a positive effect of ΔEGFR on the proliferative rate in these glioma cells. Furthermore, this property appears to be mediated through its constitutively active kinase activity, since the kinase-deficient mutant ΔEGFR protein did not confer similar enhanced proliferation.

Reduced Apoptosis Rate in U87MG.ΔEGFR Cells in Vivo and in Vivo under Serum Starvation. We next sought to determine the effect of ΔEGFR on the rate of cell death. Apoptosis was assessed using the TUNEL assay which allows a clear and reliable detection of internucleosomal DNA fragmentation characteristic of apoptotic cells (27). The TUNEL assay was applied to sections of 10% formalin-fixed, paraffin-embedded brain tumors of medium size harvested at 3–4 weeks following inoculation. All cells in control sections pretreated with DNase I revealed positive nuclear staining, whereas control cells incubated in the absence of terminal deoxynucleotidyl transferase were negative (data not shown). The AI indicating the proportion of positive cells in the TUNEL assay was the lowest in U87MG.ΔEGFR tumors (0.22%). This value was more than 4-fold lower than that seen for U87MG-derived tumors (0.94%), tumors that overexpressed the wild-type EGFR (0.90%, P < 0.01), or kinase-deficient DK (0.83%, P < 0.001; Fig. 3A). These results indicated that apoptotic cell loss was inhibited in tumor cells expressing ΔEGFR but not in the cells that overexpressed wild-type EGFR and that this inhibition was relieved when kinase activity was lost in ΔEGFR. In tissue culture, U87MG cells also underwent apoptosis when cells were completely deprived of serum for 75 h or more. Similar to the apoptosis rates observed in vivo, U87MG.ΔEGFR cells had a significantly lower AI than U87MG cells (P < 0.001), showing increased resistance to serum starvation. Neither overexpression of the kinase-deficient mutant DK (P > 0.001) nor wild-type EGFR (P > 0.01) conferred resistance to apoptosis as seen with U87MG.DK.ΔEGFR cells. In addition, stimulation of cells overexpressing wild-type EGFR with EGF had no significant effect on the apoptotic cell death rate (Fig. 3B). Taken together with data shown above, it appears that both an increased rate of cell proliferation and a decreased rate of cell death contribute to the drastically enhanced tumorigenicity of
U87MG.ΔEGFR cells. Furthermore, kinase-inactivating mutations abrogate these properties of ΔEGFR.

**Upregulation of Bcl-XL Expression in U87MG.ΔEGFR Cells.** To further characterize the underlying mechanism by which apoptosis is inhibited in U87MG.ΔEGFR cells, we investigated the expression levels of the Bcl-2 gene family of apoptosis regulators in these tumors and cells under starvation (32). Bcl-2, an apoptosis inhibitor, and Bax, an apoptosis promoter, were detectable in tumors and cells. However, the expression levels in each type of tumor or cell were similar (Fig. 4, C and D). In contrast, mRNA and protein expression of Bcl-XL, which functionally resembles the Bcl-2 protein as a potent inhibitor of apoptosis, was significantly higher (more than 3-fold) in U87MG.ΔEGFR tumors when compared with U87MG-derived tumors (Fig. 4, A and B). Bcl-XL expression level was also significantly higher in U87MG.ΔEGFR cells cultured in the absence of serum than in parental cells (about 2-fold at the protein level). Although this difference is smaller than that seen in vivo, comparison of the Bcl-XL mRNA and protein levels in U87MG.ΔEGFR and U87MG.DK clearly showed that it is due to the presence of a functional ΔEGFR. Tumors expressed only small amounts of Bcl-XS, the product of the short-form splice variant of Bcl-XL, which functions as an antagonist of Bcl-XL and Bcl-2 products, detected by reverse transcription-PCR (data not shown). This up-regulation of Bcl-XL expression was markedly reduced in the tumors of U87MG.wtEGFR and kinase-deficient DK tumors (Fig. 4, A and B). A clear inverse correlation between the expression level of Bcl-XL and Al was observed in U87MG, U87MG.ΔEGFR, U87MG.DK, and U87MG.wtEGFR (Figs. 3 and 4). These data suggested that Bcl-XL expression could be regulated through constitutively active ΔEGFR at the transcriptional level and that this up-regulation of Bcl-XL expression might play an important role in the inhibition of spontaneous apoptosis in U87MG.ΔEGFR tumors, resulting in accelerating tumor expansion.

**DISCUSSION**

We previously showed that ΔEGFR markedly enhanced the tumorigenicity of glioblastoma cells (18). However, an increased growth rate of cells was not evident when cultured in serum containing medium in vitro, leaving unresolved the question of whether the effect of ΔEGFR was primarily cell intrinsic, or due to a field effect, or a combination of the two. Here, we addressed this issue directly by mixing U87MG.ΔEGFR cells with large excesses of parental U87MG glioblastoma cells and allowing them to form tumors in the brains of nude mice. We hypothesized that if the tumorigenic effect of ΔEGFR was due to a field effect, the ratio of the two cell populations should not change in the course of tumor growth. Alternately, if ΔEGFR bestowed a cell intrinsic advantage, then cells bearing the receptor would increase in proportion. We observed an extreme form of the latter behavior: a starting ratio of U87MG.ΔEGFR to U87MG cells of 1:50,000 at injection became 5:1 in the fully grown tumor. Direct analysis of proliferation and cell death rates in tumors showed that this cell intrinsic growth advantage was due to both increased cell proliferation and reduced apoptosis of cells bearing the ΔEGFR. In vitro,
ENHANCED TUMORIGENICITY CONFERRED BY A MUTANT EGFR

Fig. 4. Up-regulation of Bcl-XL expression in U87MGΔEGFR tumors and cells. Stereotactically inoculated brain tumors were resected 3 or more weeks after implantation, immediately frozen in liquid nitrogen, and stored at −80°C. For in vitro assays, cells cultured under complete serum starvation for 48 h were harvested. Additionally, U87MG.wtEGFR cells were also stimulated with 100 ng/ml EGF at 24 h after starvation (U87MG.wtEGFR + EGF). Either total RNA or total cell lysate was extracted from the tumor tissues and cells.

A, upper gel: Northern blotting of bcl-XL expression in xenografted brain tumors and cells under serum starvation. Fifteen µg of total RNA were size fractionated in a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and probed with a human bcl-XL cDNA. Numbers below the gel, relative expression levels of bcl-XL mRNA in each tumor normalized to that of U87MG tumor. Lower gel, methylene blue staining of 28S rRNA demonstrates similar loading of RNA in each lane. B, Western blotting of Bcl-XL expression.

C, Western blotting of Bcl-2 expression. D, Western blotting of Bax expression. Numbers below the gels, expression levels of proteins in each tumor normalized to that of the U87MG tumor. Similar experiments were done more than three times, and representative blots are shown. The experiments were reproduced in three independent tumor specimens.

kD, kilodaltons.

these characteristics became evident by culturing cells in completely serum-free conditions. Reduced cell death was inversely correlated with increased expression of Bcl-XL, but not of Bcl-2 or Bax. Finally, we showed that a kinase-deficient ΔEGFR was unable to provide glioblastoma cells with a similar growth advantage, suggesting that ΔEGFR signals in a way similar to other tyrosine kinases.

Wild-type EGFR transduces mitogenic signals upon activation by binding to its specific ligands, EGF, or transforming growth factor α.

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(33). Down-regulation of EGFR occurs rapidly after activation and its signals are attenuated in the process of receptor internalization and endocytosis (34, 35). In contrast to the tight regulation of the wild-type, ΔEGFR is ligand independent and constitutively tyrosine phosphorylated (18). Our present data showing that glioblastoma cells expressing ΔEGFR had higher proliferation indexes and lower rates of apoptosis when compared to cells expressing endogenous wild-type EGFR, or cells overexpressing exogenous wild-type EGFR, suggests that these quantitative differences in receptor signaling have important physiological impact. It would indicate that sustained ligand-independent signaling of proliferation by ΔEGFR in these cells is more potent than that through overexpressed wild-type EGFR, which must be acutely stimulated in tumors in an autocrine or paracrine fashion by ligand for maximum activation, as seen in Fig. 2C (16).

In addition to stimulating proliferation, ΔEGFR also reduced apoptosis, another important determinant of net tumor growth. Interestingly, several investigators have demonstrated that proliferative activity and apoptosis rate are positively correlated in several types of malignant tumors including gliomas (23, 36) and that AI increases with higher grades of tumors (37), although the reason for this correlation remains unclear and is not invariable (38). The inhibition of cell death, in addition to augmenting tumor growth, also results in the survival of cells where abnormality would ordinarily have led to apoptosis, thereby contributing to the selective expansion of increasingly malignant cells. For example, it has been shown that oncogenically transformed cells become resistant to hypoxia-induced apoptosis and thus survive and expand clonally under hypoxic condition when they lose the wild-type p53 tumor suppressor gene or overexpress the apoptosis-inhibiting protein Bcl-2 (39). It is likely that tumor cells implanted in the brain are readily exposed to the selective pressures engendered by ischemia, malnutrition, and immune reactions which are also known to induce spontaneous apoptosis in tumors (22). Our observation showing significant suppression of apoptosis in U87MG.ΔEGFR tumors grown as xenografts in the brain as well as these cells cultured under serum starvation indicates that glioblastoma cells expressing ΔEGFR have a greater potential to survive in such environments and perhaps attain more growth factor independence. Whether this is also true in primary human brain tumors should be investigated by combining detection of ΔEGFR with the TUNEL assay in serial sections of clinical tumor specimens.

The reduction of apoptosis in U87MG.ΔEGFR tumor cells was inversely correlated with the expression of Bcl-Xl at both the mRNA and protein levels, whereas Bcl-2 and Bax expression were unchanged. The extent of Bcl-Xl up-regulation by U87MG.ΔEGFR cells was slightly lower in vitro than in vivo, suggesting that there are differences in the regulatory signals present in these two environments. Nevertheless, in both cases there was a specific increase in Bcl-Xl that correlated positively with a reduced apoptotic rate and depended on the presence of the ΔEGFR. The increase in Bcl-Xl mRNA implicates the constitutively active truncated EGFR in the transcriptional regulation of Bcl-Xl and suggests a close relationship of Bcl-Xl expression with the inhibition of apoptosis in this cell type. Further evidence for the link between ΔEGFR signaling and Bcl-Xl expression comes from experiments on the tyrosine-mutated receptors: the degree of Bcl-Xl up-regulation in these tumors was in proportion to the extent of tyrosine autophosphorylation (data not shown) and completely absent in the tumor expressing the kinase-deficient mutant. Moreover, this mechanism is postulated to be Bcl-Xl specific since neither Bcl-2 nor Bax expression was affected.

The involvement of Bcl-Xl in the suppression of glioma cell apoptosis is consistent with what is known about the function and expression of the gene. Bcl-Xl is the product of the long-form splice variant of the bcl-x gene, structurally and functionally closely resembles Bcl-2 as a potent inhibitor of cell death (24), and has been shown to prevent hypoxia-induced apoptosis more efficiently than Bcl-2 (40) and to block X-ray irradiation-induced apoptosis (41). Bcl-Xl is expressed widely in development, especially in the brain and kidney, and its expression is retained in the adult CNS unlike Bcl-2 (24, 42, 43). Expression of Bcl-Xl is also detected frequently in neuroblastoma cell lines (44) and some glioma cell lines including U87MG.4 These observations suggest a role of Bcl-Xl in CNS homeostasis and therefore make it a candidate for involvement in gliomagenesis.

The observation that the proportions of the cells changed strikingly in favor of U87MG.ΔEGFR cells between the original mixture and the final brain tumor suggests it is unlikely that ΔEGFR had positive effects on surrounding cells. Indeed, the very low final number of U87MG cells in the resultant tumors may itself require explanation in terms of the effects that the faster growing ΔEGFR-expressing variant has on its parental counterpart. For example, U87MG cells might experience limiting nutrients in the course of being overtaken by U87MG.ΔEGFR cells in vivo. Alternatively, it is possible that U87MG.ΔEGFR cells have direct cytotoxic effects on cells without this mutant receptor in vivo. Conversely, the ability of only 10 or 50 U87MG.ΔEGFR cells to survive and expand in the brain is probably dependent on the surrounding parental U87MG cells. The hypothesis that cell clonal variants growing in a tumor may be protected from the host immunosurveillance by surrounding parental cells is an intriguing one. The experiments described here could be used as a model for tumor progression by clonal evolution, where a few cells acquire an additional genetic alteration that is beneficial for their additional growth (19). By altering the properties of the parental U87MG cells and the new variant U87MG.ΔEGFR cells and studying the effect on the growth rate and composition of the resultant tumors, the interplay between the two cellular components of an evolving glioma could be elucidated.

Among the genetic alterations involved in the progression of human gial tumors, amplification and rearrangement of EGFR is considered a key event that occurs at the transition to glioblastoma multiforme (3). This notion has been supported by intensive genetic analyses of human gliomas that revealed a highly restricted occurrence of EGFR alterations in those gliomas with extreme malignancy (14—16). Our investigations of the biological effect of this receptor on glioblastoma cells show that its dual effect on proliferation and apoptosis contributes to an explanation of its pivotal role in glioma progression. It will be of importance to elucidate the nature and functions of such mutant EGFR in glioblastoma in more detail as well as to investigate their potential role in new therapeutic strategies against this intractable brain tumor.

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REFERENCES


4 Unpublished data.


A Common Mutant Epidermal Growth Factor Receptor Confers Enhanced Tumorigenicity on Human Glioblastoma Cells by Increasing Proliferation and Reducing Apoptosis

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