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

Motoo Nagane, Frank Coufal, Hong Lin, Oliver Bögler, Webster K. Cavenee, and H-J. Su Huang


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 de 2–7 EGFR). We previously demonstrated that introduction of ΔEGFR into human U87MG glioblastoma cells (U87MGΔEGFR) 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 U87MGΔEGFR cells had significantly higher labeling indexes than those 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 U87MGΔEGFR 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 U87MGΔEGFR-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 episomal 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 seen in angiogenesis and paracrine growth factor stimulation, favoring the expansion of the tumor as a whole (16, 20). Tumor 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 as 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 U87MGΔEGFR 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. 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 pronounced 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 exogenous 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 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 ATP-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 (U87MG.IΔEGFR). 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 cultivated in DMEM supplemented with 10% heat-inactivated cosmic 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) were 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) were 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) were immersed in 30% sucrose in PBS at 4°C.

In Situ (TUNEL) Labeling of Apoptotic DNA Fragmentation. In paraffin-embedded, 10% formalin-fixed tissue sections of xenografted tumors in the nude mouse brain 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 I (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 treated 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 1000–8000 cells. The rate 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 A1, 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-X, 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 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 pyrophosphate. 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-linked secondary antibodies followed by 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 × 105 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 growing to a large size in longer incubation times (more than 90% U87MG.Akp cells double-infected with luciferase and LacZ). Thus, these experiments suggest that ΔEGFR overexpression 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.wtEGR 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
Reduced Apoptosis Rate in U87MG.AEGFR Cells in Vivo and in Vitro 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 pre-treated with D Nase 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 mutant 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.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.∆EGFR cells. Furthermore, kinase-inactivating mutations abrogate these properties of ∆EGFR.

Upregulation of Bcl-X<sub>L</sub> Expression in U87MG.∆EGFR Cells. To further characterize the underlying mechanism by which apoptosis is inhibited in U87MG.∆EGFR 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-X<sub>L</sub>, which functionally resembles the Bcl-2 protein as a potent inhibitor of apoptosis, was significantly higher (more than 3-fold) in U87MG.∆EGFR tumors when compared with U87MG-derived tumors (Fig. 4, A and B). Bcl-X<sub>L</sub> expression level was also significantly higher in U87MG.∆EGFR 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-X<sub>L</sub> mRNA and protein levels in U87MG.∆EGFR and U87MG.DK clearly showed that it is due to the presence of a functional ∆EGFR. Tumors expressed only small amounts of Bcl-X<sub>S</sub>, the product of the short-form splice variant of Bcl-X<sub>L</sub> which functions as an antagonist of Bcl-X<sub>L</sub> and Bcl-2 products, detected by reverse transcription-PCR (data not shown). This up-regulation of Bcl-X<sub>L</sub> 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-X<sub>L</sub> and Al was observed in U87MG, U87MG.∆EGFR, U87MG.DK, and U87MG.wtEGFR (Figs. 3 and 4). These data suggested that Bcl-X<sub>L</sub> expression could be regulated through constitutively active ∆EGFR at the transcriptional level and that this up-regulation of Bcl-X<sub>L</sub> expression might play an important role in the inhibition of spontaneous apoptosis in U87MG.∆EGFR tumors, resulting in accelerating tumor expansion.

DISCUSSION

We previously showed that ∆EGFR 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 ∆EGFR 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.∆EGFR 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 ∆EGFR was due to a field effect, the ratio of the two cell populations should not change in the course of tumor growth. Alternately, if ∆EGFR 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.∆EGFR 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 ∆EGFR. In vitro,
ENHANCED TUMORIGENICITY CONFERRED BY A MUTANT EGFR

Fig. 4. Up-regulation of Bcl-XL expression in U87MG ΔEGFR 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. Equal amounts of tumor or cell lysates were subjected to 12.5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-human Bcl-XL polyclonal antibody. 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.
expression of the gene. Bcl-XL is the product of the long-form splice proportion to the extent of tyrosine autophosphorylation (data not shown) and completely absent in the tumor expressing the kinase 43). Down-regulation of EGFR occurs rapidly after activation and its expressions 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 glial tumors, amplification and rearrangement of EGF 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 EGF 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.

ACKNOWLEDGMENTS

We thank Xiang-Dong Ji and Donna Harclerode (PharMingen, Inc.) for preparing sections for staining.

REFERENCES


4 Unpublished data.
breaks associated with apoptosis in human brain tumors. Virchows Arch., 427:

22. Kerr, J. F., Winterford, C. M., and Harmon, B. V. Amplified and
expression and possible rearrangement of EGF receptor gene in primary human brain

23. Nakagawa, S., Shiraishi, T., Kihara, S., and Tabuchi, K. Detection of DNA strand
breaks associated with apoptosis in brain human tumors. Virochim Arch., 427:

24. Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka,
L. A., Mao, X., Nunez, G., and Thompson, C. B. bel-x, a bel-2-related gene that


encoding alkaline phosphatase confirms clonal boundary assignment in lineage anal-

27. Gavriel, Y., Sherman, Y., and Ben-Sasson, S. A. Identification of programmed cell
death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol., 119:

28. Herrin, D. L. and Schmidt, G. W. Rapid, reversible staining of northern blots prior to

cycle analysis of a cell proliferation-associated human nuclear antigen defined by the

demonstration of S-phase cells by anti-bromodeoxyuridine monoclonal antibody in

significance of the proliferative potential of intracranial gliomas measured by bro-

1—6, 1994.

33. Todaro, G. J., Frying, C., and De Larco, J. E. Transforming growth factors produced
by certain human tumor cells: polypeptides that interact with epidermal growth factor

Ligand-induced transformation by a noninternalizing epidermal growth factor recep-

Endooyisis and lysosomal targeting of epidermal growth factor receptors are medi-
ated by distinct sequences independent of the tyrosine kinase domain. J. Biol. Chem.,

36. Leocinzi, L., Del Vecchio, M. T., Meghna, M., Barbini, P., Galieni, P., Pileri, S.,
Sabattini, E., Gherlinzoni, F., Tosi, P., Kraft, R., and et al. Correlations between
apoptotic and proliferative indices in malignant non-Hodgkin’s lymphomas. Am. J.

37. Kerr, K. M., and Lamb, D. Actual growth rate and tumour cell proliferation in human

38. Schiffer, D., Cavalla, P., Chio, A., Giordana, M. T., Marino, S., Mauro, A., and
Miguell, A. Tumor cell proliferation and apoptosis in medulloblastoma. Acta Neu-

death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol., 119:

cycle analysis of a cell proliferation-associated human nuclear antigen defined by the

41. Han, Z., Chanerjee, D., He, D. M., Early, J., Pantazis, P., Wyche, J. H., and
Prevc, J. H., O’Shea, S., Boise, L. H., Thompson, C. B., and et al. Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. Nature (Lond.), 374:

42. Gonzalez-Garcia, M., Garcia, I., Ding, L., O’Shea, S., Boise, L. H., Thompson, C. B.,
and Nunez, G. bel-x is expressed in embryonic and postnatal neural tissues and
functions to prevent neuronal cell death. Proc. Natl. Acad. Sci. USA, 92: 4304—4308,
1995.

43. Krajewski, S., Krajewska, M., Shabaik, A., Wang, H. G., Irie, S., Fong, L., and Reed,
J. C. Immunohistochemical analysis of in vivo patterns of Bel-x expression. Cancer

44. Dole, M. G., Jasty, R., Cooper, M. J., Thompson, C. B., Nunez, G., and Castle, V. P.
A Common Mutant Epidermal Growth Factor Receptor Confers Enhanced Tumorigenicity on Human Glioblastoma Cells by Increasing Proliferation and Reducing Apoptosis

Motoo Nagane, Frank Coufal, Hong Lin, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/56/21/5079

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.