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Genetics

A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity

(tumor progression/glioblastoma)

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ABSTRACT **The development and neoplastic progression of human astrocytic tumors appears to result through an accumulation of genetic alterations occurring in a relatively defined order. One such alteration is amplification of the epidermal growth factor receptor (EGFR) gene. This episomal amplification occurs in 40–50% of glioblastomas, which also normally express endogenous receptors. Moreover, a significant fraction of amplified genes are rearranged to specifically eliminate a DNA fragment containing exons 2–7 of the gene, resulting in an in-frame deletion of 801 bp of the coding sequence of the extracellular domain. Here we used retroviral transfer of such a mutant receptor (de 2–7 EGFR) into glioblastoma cells expressing normal endogenous receptors to test whether the mutant receptor was able to augment their growth and malignancy. Western blotting analysis showed that these cells expressed endogenous EGFR of 170 kDa as well as the exogenous de 2–7 EGFR of 140–155 kDa. Although holo-EGFRs were phosphorylated on tyrosine residues only after exposure of the cells to ligand, de 2–7 EGFRs were constitutively phosphorylated. In tissue culture neither addition of EGF nor expression of the mutant EGFR affected the rate of cell growth. However, when cells expressing mutant EGFR were implanted into nude mice subcutaneously or intracerebrally, tumorigenic capacity was greatly enhanced. These results suggest that a tumor-specific alteration of the EGFR plays a significant role in tumor progression perhaps by influencing interactions of tumor cells with their microenvironment in ways not easily assayed *in vitro*.**

The clonal evolution of neoplasms toward increasing malignancy has been ascribed to the accumulation of genetic alterations that are advantageous to growth (1). This hypothesis has gained experimental support in colorectal cancer (2), squamous carcinoma of the cervix (3), lymphoid tumors (4), and gliomas (5). In the latter case, a series of events occurring in a relatively ordered fashion have been identified and include loss of heterozygosity for chromosome 17p (6, 7), mutation of the p53 gene (5), deletions of the interferon gene cluster on chromosome 9p (8), amplification and alteration of the epidermal growth factor receptor (EGFR) gene (9, 10), and monosomy of chromosome 10 (11, 12).

The involvement of EGFR in human cancer has been inferred from its ability to confer ligand-dependent transformation to NIH 3T3 cell transfectants (13) and by its frequent gene amplification and overexpression in carcinomas of the breast (14), ovary (15), cervix (15), kidney (15), and squamous cells (16). In glial tumors of the central nervous system, such alterations are restricted to grade III (anaplastic astrocytoma) and, especially, grade IV (glioblastoma multiforme) disease (10, 17–19). Several clinical and histopathological studies have shown that EGFR gene amplification is related to a shorter interval to relapse and poorer survival (17, 20, 21). Nearly half of tumors examined had significantly amplified EGFR genes (10, 18, 19) and, of these, the majority also show rearrangements of the gene resulting in alterations of its transcript so that such gliomas express both wild-type endogenous EGFR as well as the episomal mutant form. The most common of the rearrangements are genomic alterations leading to deletion of exons 2–7 in the EGFR mRNA, which causes an in-frame truncation of 801 bp in the extracellular domain of the molecule (9, 10, 22–24). Introduction of such a cDNA isolated from a glioma into murine NIH 3T3 cells resulted in expression of a constitutively phosphorylated membrane-associated 150-kDa receptor species, which caused weak ligand-independent cell transformation (25).

Despite the common occurrence of these EGFR aberrations in gliomas, virtually all cell lines derived from them lose these features in tissue culture (26), suggesting either a growth disadvantage *in vitro* or a selection for EGFR overexpression *in vivo*. To study the biological effects of a mutant EGFR that lacks exons 2–7 (de 2–7 EGFR), we used retroviral transfer to achieve high-level expression of this protein in glioblastoma cells expressing wild-type endogenous receptor. In glioblastoma cells de 2–7 EGFR exhibited constitutively activated self-phosphorylation and exerted a pronounced enhancement of tumorigenicity *in vivo*.

MATERIALS AND METHODS

Construction and Production of Mutant EGFR Retroviral Vectors. Mutant EGFR (de 2–7 EGFR) was constructed from the full-length EGFR cDNA clone pRC/EGFR (a gift of M. G. Rosenfeld, Howard Hughes Medical Institute) by eliminating the sequences of exons 2–7. Two DNA fragments corresponding to nucleotides 83–274 and 1076–1357 (27) were synthesized by PCR using two pairs of primers, TCAGATCTCTAGAGAGC (sense)/AGACCTCCTTTTCTTTCCGGAGG (antisense) and AAGAAGTGTCCCATTAATTATGTGGT (sense)/AGGAGACCTAGGTGTCC (antisense), respectively. The first PCR product was digested with *Xba* I and *Stu* I and the second fragment was digested with *Ase* I, blunt-ended with the Klenow fragment of DNA polymerase, and digested with *Bam*HI. The two resulting fragments were then ligated and subcloned into pBluescript SK− (Stratagene). The *Xba* I/*Dra* I EGFR fragment was isolated from the plasmid and ligated with the *Dra* I/*Hin*dIII fragment from pRC/EGFR to create de 2–7 EGFR, which was cloned into pBluescript SK− and verified by DNA sequencing. De 2–7 EGFR was transferred to a retroviral vector, pLRNL (28), and introduced into ψ-2 cells with Lipofectin (GIBCO/BRL).

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Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FACS, fluorescence-activated cell sorter.

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Virus was collected 48 hr later and was used to infect PA317. G418-resistant PA317 cells were assayed for mutant EGFR expression by fluorescence-activated cell sorter (FACS) analysis; cells staining positively were sorted, analyzed for de 2–7 EGFR expression by Western blotting analysis, and used as virus-producing stocks.

FACS. The human glioblastoma cell line U87MG (29) was infected with virus and selected in a medium containing 400 μg of G418 per ml according to described procedures (28); 5×10^5 cells in 200 μl of staining buffer [phosphate-buffered saline (PBS)/1% fetal bovine serum (FBS)/0.1% sodium azide] were exposed to anti-EGFR monoclonal antibody Ab-1 (clone 528) (30) or Ab-5 (clone EGFR.1) (31) (both obtained from Oncogene Science) for 30–60 min and then to fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody (PharMingen) for 30–60 min. Stained cells were analyzed with a FACSort (Becton Dickinson); for cell sorting, sodium azide was omitted from the staining buffer, and cells were sorted with a FACStar (Becton Dickinson).

Western Blotting. Cells (4×10^4 cells per well) in a 12-well plate were cultured overnight in medium containing 10% FBS (PA317 and PA317 virus stock cells) or 2% dialyzed FBS (U87MG and U87MG.ΔEGFR). For antibody and ligand treatments, cells were washed with PBS and treated with anti-EGFR antibody Ab-1 (5 μg/ml) for 3 hr at 37°C, and then 100 ng of EGF (Collaborative Biomedical Products, Bedford, MA) per ml for 5 min at room temperature. Cells were washed with PBS and lysed in 200 μl of lysis buffer [0.064 M Tris·HCl, pH 6.8/1.28% SDS/12.8% (vol/vol) glycerol/1.28% 2-mercaptoethanol/0.25% bromphenol blue] and then boiled. Frozen tumor samples were homogenized in a Dounce homogenizer containing lysis buffer, boiled, and centrifuged to remove cell debris. The lysates were electrophoresed through SDS/7% polyacrylamide gels and the separated proteins were transferred to nitrocellulose membranes before being probed with anti-EGFR rabbit polyclonal antibody 1964 (32), or anti-phosphotyrosine [anti-Tyr(*P*)] mouse monoclonal antibody PY20 (Transduction Laboratories, Lexington, KY), as described (33).

Tumorigenicity. For subcutaneous inoculation, U87MG (1×10^6 cells) or U87MG.ΔEGFR (1×10^6 or 2×10^5 cells) was suspended in 0.2 ml of PBS and injected simultaneously into the left or right flanks, respectively, of 4- to 5-wk-old female nude mice of BALB/c background. The growing tumors were measured twice a week with a caliper, and tumor volumes were calculated using width (*a*) and length (*b*) measurements ($a^2b/2$, where $a < b$) (34). For intracerebral stereotactic implantation, 5×10^4–5×10^5 cells in 5 μl of PBS were inoculated into the corpus striatum in the right hemisphere (2.5–3.0 mm deep; 1 mm anterior and 1.8 mm lateral to the bregma) of the nude mouse brain (35). Brains were removed at various time points, embedded in OCT compound (Miles), frozen in liquid nitrogen, and stored at −80°C. Thin cryostat sections (5–7 μm) were stained with hematoxylin, and tumor size was microscopically determined.

Immunohistochemistry. Cryostat sections (5 μm) were air-dried overnight, washed with PBS, and fixed in cold acetone for 10 min. Endogenous peroxidase activity was quenched with 2% hydrogen peroxide in PBS, and nonspecific binding sites were blocked with 1% bovine serum albumin for 15 min at room temperature. The sections were incubated with the anti-EGFR antibody Ab-1 (5 μg/ml) for 1 hr at room temperature. Biotinylated horse anti-mouse IgG(H+L) antibody and avidin/biotin/horseradish peroxidase were then applied to the sections as recommended (Vector Laboratories). Immunoreactivity was revealed by adding the peroxidase substrate 3,3′-diaminobenzidine tetrahydrochloride (Vector Laboratories), and sections were counterstained with hematoxylin.

RESULTS

Expression of Mutant EGFR in Glioblastoma Cells. U87MG cells were infected with the amphotropic virus, which carries a neomycin-resistance gene and an EGFR gene deleted for exons 2–7. Cells were selected for G418 resistance and assessed for the presence of EGFR on their cell surfaces by FACS analysis using the Ab-1 monoclonal antibody, which recognizes an epitope shared by wild-type and de 2–7 EGFRs; 85% of the cells were more heavily stained than the parental U87MG cells, indicating expression of the exogenous gene; the 7% of cells that were most brightly stained were sorted and the population (U87MG.ΔEGFR) was characterized further by FACS analysis. U87MG.ΔEGFR reacted equally as well as parental cells with the monoclonal antibody Ab-5, which reacted with wild-type but not de 2–7 EGFR, suggesting that this population expressed levels of the endogenous EGFR similar to that of parental cells (Fig. 1*A*). However, when cells were stained with Ab-1, the mean of fluorescence intensity for U87MG.ΔEGFR was 4–5 times higher than that of parental cells (Fig. 1*B*). By careful concordant comparisons, this level of expression was equivalent to that of the A431 squamous cell carcinoma line, which has been shown to express $1–3 \times 10^6$ EGFR molecules on its surface (36–38). Moreover, the U87MG.ΔEGFR population was relatively homogeneous in its expression as demonstrated by the narrow fluorescence intensity profiles. These characteristics remained stable in the presence of G418 selection over many months of culture.

Self-phosphorylation of EGFR occurs rapidly after ligand binding and functions in assembly of signaling components that contain SH2 domains (39). The transforming protein

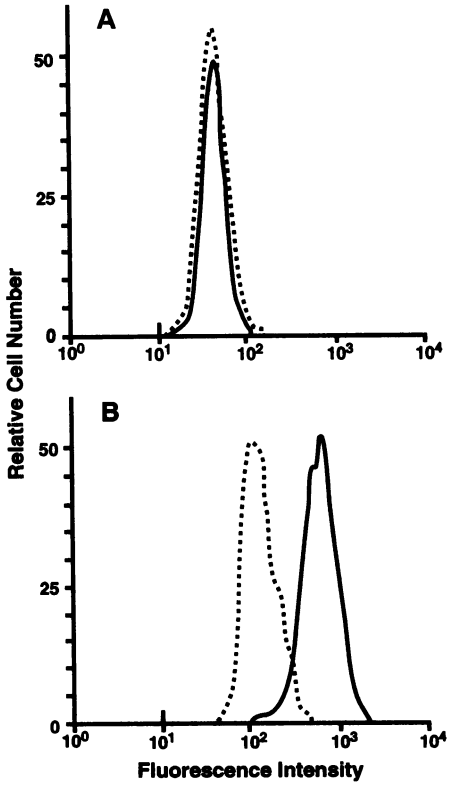

FIG. 1. Surface expression of endogenous and de 2–7 EGFR in U87MG and U87MG.ΔEGFR cells as determined by FACS analysis. U87MG (dotted lines) and U87MG.ΔEGFR (solid lines) were stained with anti-EGFR monoclonal antibody Ab-5 (*A*), which recognizes endogenous EGFR exclusively, or Ab-1 (*B*), which reacts with both endogenous and de 2–7 EGFR. Bars on the abscissa in *A* and *B* represent maximum staining of cells in the absence of primary antibody.

v-erbB is constitutively active and self-phosphorylated (40, 41). To determine the activation state of de 2–7 EGFR, U87MG and U87MG.ΔEGFR cells were treated without or with EGF, and receptor self-phosphorylation was measured with monoclonal anti-Tyr(*P*) antibody. Probing Western blots with anti-EGFR antibody showed that parental U87MG and U87MG.ΔEGFR expressed the same level of 170-kDa endogenous EGFR (Fig. 2*A*, lanes 1 and 5). Endogenous EGFR was tyrosine phosphorylated only when ligand was added to the medium (Fig. 2*B*, lanes 2 and 6). Anti-EGFR antibody also detected molecules of 140 and 150–155 kDa, which were unique to U87MG.ΔEGFR cells, and corresponded to posttranslationally modified de 2–7 EGFR. The ratio of 170-kDa species to 140- to 155-kDa molecules was similar to that observed in FACS analysis, suggesting that the majority of de 2–7 EGFRs produced in U87MG.ΔEGFR are expressed on their surface. The 150- to 155-kDa molecules reacted with anti-Tyr(*P*) antibody but, in contrast to endogenous EGFR, their phosphorylation was constitutive and not dependent on exogenous ligand (Fig. 2*B*, lanes 5 and 6). To rule out the possibility that constitutive phosphorylation of the 150- to 155-kDa proteins was due to residual ligand in the culture medium, an anti-EGFR antibody that blocks ligand binding to the receptor and subsequent autophosphorylation of the receptor was used (42). The effectiveness of this antibody is demonstrated for parental U87MG cells in Fig. 2*B* (lanes 3 and 4). However, the antibody was not able to inhibit phosphorylation of the 150- to 155-kDa molecules in U87MG.ΔEGFR cells, even though phosphorylation of the endogenous 170-kDa species in these cells occurred in response to EGF and was blocked by exposure to the antibody (lanes 7 and 8).

The inability of EGF to stimulate tyrosine self-phosphorylation of de 2–7 EGFR suggested that removal of exons 2–7 disrupted ligand binding. At least one point of contact between receptor and ligand, Tyr^{101} (43), located in exon 4 (44) is lost. To examine this directly, levels of ^{125}I-labeled EGF binding to U87MG and U87MG.ΔEGFR cells were compared. Both the K_d and B_{max} values were similar (K_d = 3.8 and 3.2 nM; B_{max} = 2.1 and 2.3 × 10^5 EGF binding sites per cell, for U87MG and U87MG.ΔEGFR cells, respectively). Because the two cells contain similar amounts of holo-EGFR (Fig. 2*A*), the observation that similar amounts of ligand were bound argues that EGF does not bind to the 4- to 5-fold excess amount of de 2–7 EGFR present on the surface of U87MG.ΔEGFR cells.

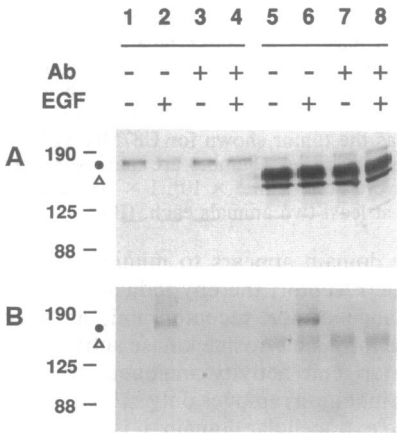

FIG. 2. Western blotting analysis of EGFR expression and tyrosine phosphorylation in U87MG (lanes 1–4) and U87MG.ΔEGFR (lanes 5–8). Cells were treated (+) or not (−) with the monoclonal antibody Ab-1, which is a competitive antagonist for EGF binding, for 3 hr at 37°C and incubated in the presence (+) or absence (−) of EGF (100 ng/ml) for 5 min at room temperature before being lysed in SDS-containing buffer. (*A*) Blots were probed with anti-EGFR polyclonal antibody 1964. (*B*) Blots were probed with anti-phosphotyrosine monoclonal antibody PY20. Endogenous EGFR of 170 kDa (●) and tyrosine-phosphorylated proteins of 150–155 kDa (△) are indicated on the left. Numbers are molecular mass markers (kDa). The experiment was reproduced four separate times.

Effect of Mutant EGFR on Cell Growth *in Vitro*. Because de 2–7 EGFR was expressed at a high level in glioblastoma cells and was constitutively active as assessed by tyrosine phosphorylation, we analyzed its influence on cellular growth rate. Fig. 3*A* shows that the growth of these two cell populations was identical when they were cultured in medium containing 10% FBS. Since 2% dialyzed serum was the minimum requirement to sustain growth, we cultured U87MG and U87MG.ΔEGFR cells in medium containing 2% dialyzed FBS and various concentrations of the ligands, EGF, or transforming growth factor α through a concentration range of 0.1–100 ng/ml. In no case did either ligand affect the growth rates of either cell population and, although U87MG.ΔEGFR grew slightly faster than U87MG in medium containing 2% dialyzed serum, their saturation densities were the same (Fig. 3*B*). To assume that the retroviral vector itself was not playing a role in these results, we also infected U87MG cells with a similar construct expressing luciferase. These cells and the parental cells had identical growth characteristics.

Effect of Mutant EGFR on Tumorigenicity *in Vivo*. We next tested whether the expression of a mutant EGFR had any effect on the ability of glioblastoma cells to form tumors in animals. U87MG.ΔEGFR cells grew remarkably faster than parental U87MG cells (or U87MG cells infected with the luciferase viral construct) when they were implanted subcutaneously into the flanks of nude mice (Fig. 4). U87MG.ΔEGFR caused palpable tumors in 1 wk when 1 × 10^6 cells were inoculated, whereas the same number of parental U87MG cells required 3 wk to grow to a similar extent; at 1 × 10^6 cell inoculation, U87MG.ΔEGFR and U87MG cells required 2 and 5 wk, respectively, to form tumor masses of 1 cm^3. Even when the number of U87MG.ΔEGFR cells implanted was reduced 5-fold to 2 × 10^5, they still grew more vigorously than U87MG cells, as shown in Fig. 4.

To determine whether this growth advantage had site specificity, the glioblastoma cells were stereotactically implanted into the brains of nude mice. Cells (2 × 10^5) were inoculated and after 2 wk, three of three animals that received U87MG.ΔEGFR cells had tumors of 5 mm diameter or greater, whereas only one of five mice implanted with U87MG cells developed a tumor that had a diameter of 2 mm. Examples are shown in Fig. 5. Four animals that received 5 × 10^5 U87MG.ΔEGFR cells died within 4 wk after implan-

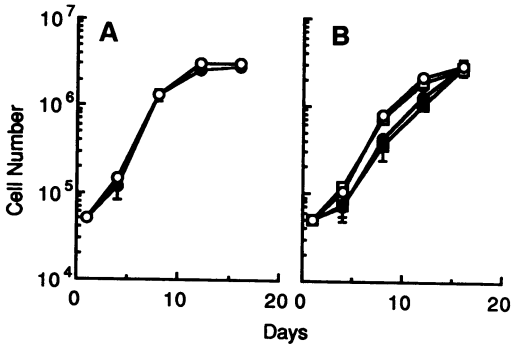

FIG. 3. Lack of *in vitro* effects on growth of U87MG cells expressing de 2–7 EGFR. U87MG (● and ■) and U87MG.ΔEGFR (○ and □) cells were grown in 10% FBS (*A*) or 2% dialyzed FBS (*B*) in the presence (■ and □) or absence (● and ○) of EGF (100 ng/ml). Cell numbers were determined at 3- to 4-day intervals and are shown as means ± SD of triplicate samples. Similar experiments were done four separate times.

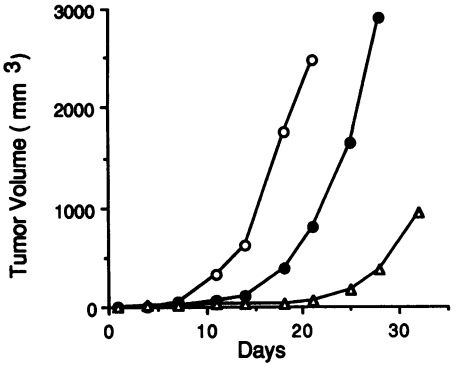

FIG. 4. Effect of de 2–7 EGFR expression on U87MG cell tumorigenicity in subcutaneous implantation. Either 1×10^6 (○) or 2×10^5 (●) U87MG.ΔEGFR cells were injected into the right flank of nude mice while 1×10^6 (△) U87MG cells were inoculated into the left flank of the same animals. Tumor size was quantitated at the indicated times. U87MG, $n = 10$; U87MG.ΔEGFR, $n = 5$. Similar experiments were done three separate times.

tation due to the overgrowth of tumors, which attained sizes of >10 mm in diameter, while four mice inoculated with the same number of parental U87MG cells or four animals implanted with U87MG cells infected with retroviral luciferase construct formed small tumors of <4 mm in diameter. Another group of animals that received 5×10^4–1×10^5 cells also showed a similar outcome after 3 wk: two of two mice that received U87MG.ΔEGFR cells developed tumors of >5 mm in diameter. None of the five animals implanted with parental U87MG cells produced tumors of >1 mm. Thus, the expression of de 2–7 EGFR in glioblastoma cells that also express endogenous EGFR conferred a substantial growth advantage *in vivo*.

Previous studies showed that amplification of EGFR expression is an unstable situation *in vitro* (26). To confirm that the enhanced tumorigenicity of U87MG.ΔEGFR cells was due to the effect of the mutant EGFR, we analyzed EGFR expression in tumors excised from the implanted animals. Western blotting analysis showed that tumors obtained from subcutaneous and intracerebral implantations of U87MG.ΔEGFR expressed endogenous EGFR of 170 kDa as well as smaller species of 140 and 150–155 kDa, in which the 150- to 155-kDa receptors were tyrosine-phosphorylated and the ratio of endogenous to mutant receptor was comparable to that of U87MG.ΔEGFR grown *in vitro* (as in Fig. 2), indicating stability of expression of the mutant EGFR (data not shown). Moreover, the expression of EGFRs in U87MG.ΔEGFR and U87MG tumor cells was homogeneous as shown by the immunohistochemical staining of cryostat sections (Fig. 5).

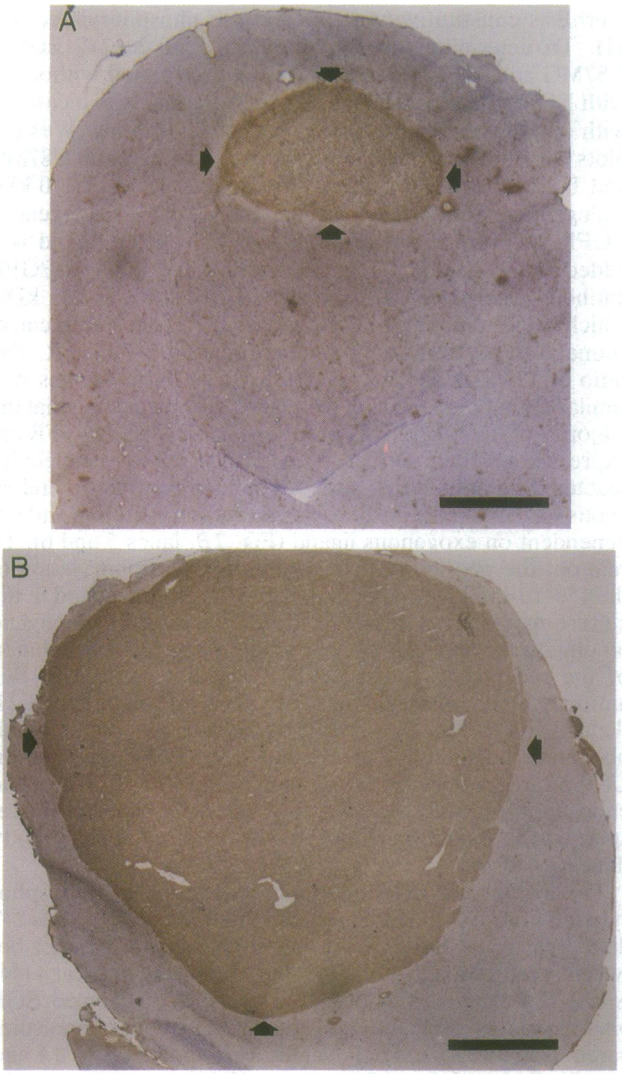

FIG. 5. Effect of de 2–7 EGFR on U87MG tumorigenicity in intracerebral implantation. Either 2×10^5 U87MG (*A*) or 2×10^5 U87MG.ΔEGFR (*B*) cells were stereotactically inoculated into the brains of nude mice. Two weeks postimplantation, brains were resected for analysis. Frozen sections were stained with the anti-EGFR antibody Ab-1 and counterstained with hematoxylin. The small tumor shown for U87MG in *A* is the largest obtained in the series, whereas the tumor shown for U87MG.ΔEGFR in *B* was of average size in the series. Tumors are marked by arrows. Similar experiments were done with 5×10^4, 1×10^5, and 5×10^5 cells of both types in at least two animals each. (Bars = 1 mm.)

DISCUSSION

Expression of a mutant EGFR, which occurs commonly in human glial tumors, enhances the tumorigenic activity of U87MG glioblastoma cells *in vivo* in the absence of detectable *in vitro* growth advantages. Holo-EGFR is activated by ligand binding, which causes rapid dimerization (45), conformational change (46), activation of its intrinsic protein-tyrosine kinase activity, and autophosphorylation (47). Binding of ligand to the receptor also induces receptor internalization, downregulation, and attenuation of signaling (48). Thus, EGFR activities are normally under stringent positive as well as negative regulation. Disruption of the control process by mutations that either affect ligand-dependent tyrosine kinase activation mechanisms or impair ligand-induced receptor internalization may then lead to a constitutive elevation of receptor activity (48, 49). The mutant receptor we studied here may represent the former case, since it is constitutively tyrosine phosphorylated. Deletion of the portion encoded by exons 2–7 of the extracellular domain appears to mimic the effect of ligand binding to the receptor, thereby inducing the active conformational change of the receptor and, as a consequence, activating the intrinsic tyrosine kinase activity and so enhancing their tumorigenic activity analogous to v-erbB (40, 41). Because this mutation removes only ≈1/4th of the N-terminal portion of the extracellular domain, it is also possible that this form of EGFR is able to interact with unknown ligands *in vivo* or that it forms heterodimers with other members of the EGFR family, thereby eliciting the biological effects. This could explain why expression of the mutant EGFR in U87MG cells influences their phenotype *in vivo* but not *in vitro*. Nevertheless, previous findings have shown that a mutant EGFR derived by a large deletion of virtually the entire extracellular domain of the receptor was constitutively active and capable of transforming established rodent fibroblasts to a tumorigenic phenotype (50), suggesting by analogy that the enhancing effect on tumorigenicity observed results, at least partially,

from ligand-independent activation of the receptor. This is further supported by the observation that mutant EGFR in tumors isolated from implanted animals displayed constitutive tyrosine self-phosphorylation patterns similar to those of the cells cultured *in vitro*.

In spite of its remarkable effect *in vivo*, expression of the mutant EGFR did not influence the growth rate *in vitro*. Perhaps this is because U87MG is a fully transformed cell line so that its *in vitro* responses are no longer affected by the added stimulation provided by the constitutive phosphorylation of the mutant receptor. Although EGF activates holo receptors in these cells, EGF did not influence the rate of cell growth. We have expressed high amounts of wild-type EGFR in U87MG and found this to have little effect on *in vitro* growth. Yamazaki *et al.* (25) have also demonstrated that overexpression of a similarly mutated EGFR in NIH 3T3 cells only slightly affects the cellular morphology regardless of the presence of EGF, while expression of a high level of wild-type EGFR in the same cells resulted in alterations in cellular morphology and the ability of the cell to form foci in a ligand-dependent manner (25). Effects on growth clearly depend on cell type and the presence of other mutational events.

Amplification and rearrangement of the EGFR gene occurs almost exclusively at the latest stage of the glioma malignancy process, and our results argue for a strong selection for cells carrying such receptors. Since this selection takes place *in vivo* but not *in vitro*, perhaps the mutant receptor plays a role in the interactions of tumor cells with their environment rather than directly on cell growth. The late stage of glioma at which this occurs suggests several potential processes that may be influenced in this way. Tumors at this point are dependent on neovascularization and perhaps the signals transduced by the mutant EGFR enhance the ability of the tumor cells to induce this process. At this stage, the tumors also become increasingly necrotic and perhaps the tumor cells that carry these mutant receptors are better able to survive in these ischemic conditions. Whatever the case, the present studies provide an experimentally malleable system within which to delineate this particular type of *in vivo* growth advantage.

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