

Radiosensitization of Malignant Glioma Cells through Overexpression of Dominant-Negative Epidermal Growth Factor Receptor¹

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ABSTRACT

The epidermal growth factor receptor (EGFR) plays an important role in neoplastic growth control of malignant gliomas. We have demonstrated that radiation activates EGFR Tyr-phosphorylation (EGFR Tyr-P) and the proliferation of surviving human carcinoma cells, a likely mechanism of accelerated cellular repopulation, a major cytoprotective response after radiation. We now investigate the importance of radiation-induced activation of EGFR on the radiosensitivity of the human malignant glioma cells U-87 MG and U-373 MG. The function of EGFR was inhibited through a genetic approach of transducing cells with an Adenovirus (Ad) vector containing dominant-negative (DN) EGFR-CD533 (Ad-EGFR-CD533) at efficiencies of 85–90%. The resulting cells are referred to as U-87-EGFR-CD533 and U-373-EGFR-CD533. After irradiation at 2 Gy, both of the cell lines exhibited a mean 3-fold increase in EGFR Tyr-P. The expression of EGFR-CD533 completely inhibited the radiation-induced activation of EGFR. In clonogenic survival assays after a single radiation exposure, the radiation dose for a survival of 37% (D_{37}) for U-87-EGFR-CD533 cells was 1.4- to 1.5-fold lower, relative to cells transduced with AdLacZ or untransduced U-87 MG cells. This effect was amplified with repeated radiation exposures (3×2 Gy) yielding a D_{37} ratio of 1.8–2.0. In clonogenic survival studies

with U-373 MG cells, the radiosensitizing effect of EGFR-CD533 was similar. Furthermore, *in vivo* studies with U-87 MG xenografts confirmed the effect of EGFR-CD533 on tumor radiosensitization (dose enhancement ratio, 1.8). We conclude that inhibition of EGFR function via Ad-mediated gene transfer of EGFR-CD533 results in significant radiosensitization. As underlying mechanism, we suggest the disruption of a major cytoprotective response involving EGFR and its downstream effectors, such as mitogen-activated protein kinase. The experiments demonstrate for the first time that radiosensitization of malignant glioma cells through disruption of EGFR function may be achieved by genetic therapy approaches.

INTRODUCTION

RTKs³ of the ErbB family and related plasma membrane receptors have been identified as critical components facilitating autocrine growth regulation of carcinoma and malignant glioma cells that are typically the result of coordinate coexpression of GFs and their receptors (1–4). The activation of RTKs and stimulation of downstream signaling pathways that mediate mitogenesis have been identified as the underlying mechanism (4). Among the ErbB RTKs, EGFR has been extensively studied including its involvement in the neoplastic progression of human carcinoma and malignant glioma cells (3, 5). EGFR, a M_r 170,000 transmembrane glycoprotein, contains a GF binding site in the extracellular NH_2 -portion of the molecule and an intracellular Tyr kinase domain in the cytoplasmic COOH-terminal portion (2, 3). The phosphorylation of Tyr, Ser, and Thr residues in the cytoplasmic portion are critical for its activation and downstream signaling (4, 6). As has been demonstrated for malignant gliomas and carcinomas, EGFR amplification and/or genetic modification, *e.g.*, truncation, is critically linked to neoplastic progression (7, 8).

We have recently demonstrated a role for EGFR in cellular responses to single and repeated ionizing Rad exposures (6, 9–11). The activation of EGFR by Rad resembles that of GFs with immediate EGFR activation, as reflected by increased phosphorylation of defined Tyr residues in the cytoplasmic

Received 5/26/00; revised 12/6/00; accepted 12/18/00.

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¹ Supported by USPHS Grants PO1 CA72955 and R01 CA65896 (to R. K. S.-U.) and the Deutsche Krebshilfe, Dr. Mildred Scheel Stiftung, fellowship support (to G. L.).

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³ The abbreviations used are: RTK, receptor tyrosine kinase; EGF, epidermal growth factor; EGFR, EGF receptor; EGFR Tyr-P, EGFR Tyr phosphorylation; Ad, adenovirus; DN, dominant negative; Ad-EGFR-CD533, Ad-containing dominant-negative EGFR-CD533; AdLacZ, Ad expressing the bacterial *lacZ* reporter gene; MAPK, mitogen-activated protein kinase; mAb, monoclonal antibody; GF, growth factor; β -gal, β -galactosidase; pfu, plaque-forming unit(s); MOI, multiplicity/multiplicities of infection; D_{37} , radiation dose for a survival of 37%; Rad, radiation; FBS, fetal bovine serum; Ab, antibody; mAb, monoclonal Ab; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

domain (9, 11, 12). The consequence of EGFR activation includes the stimulation of downstream kinases, such as MAPK (11). In our detailed characterization of Rad effects along the EGFR-MAPK signaling cascade (4), we have linked the activation of EGFR to a cellular proliferation response (4, 10, 11, 13, 14) and concluded that EGFR mediates Rad-induced proliferation in surviving cells. This represents a likely mechanism underlying Rad-induced accelerated cellular repopulation (4, 6, 10). Because accelerated tumor cell proliferation counteracts the cytotoxic effects of Rad, it has to be viewed as a major cytoprotective response (4, 6). Thus, disruption of this response may be one mechanism of radiosensitizing tumor cells.

Considering the poor response of human malignant gliomas, especially glioblastomas, to radiotherapy, we have investigated the importance of Rad-induced activation of EGFR on the radiosensitivity of human malignant glioma cells. For this, we have used a genetic approach by transducing cells with DN EGFR-CD533, which lacks 533 amino acids of the cytoplasmic domain responsible for mitogenic and transforming activities of the receptor (15). We have previously shown that overexpression of EGFR-CD533 completely abrogates Rad-induced activation of EGFR in human carcinoma cells with secondary complete suppression of the MAPK and Rad-induced proliferation responses (4, 6, 11, 13). Therefore, we now investigate whether inhibiting EGFR function through overexpression of EGFR-CD533 results in radiosensitization of malignant glioma cells. In line with our interest in genetic therapy approaches, the effects of overexpression of EGFR-CD533 were studied after *in vitro* Ad transduction of two human malignant glioma cell lines and also after Ad infusion in U-87 MG tumor xenografts.

MATERIALS AND METHODS

Cell Lines and Reagents. Two human glioma cell lines were used in the present study; U-373 MG, originally isolated from a human anaplastic astrocytoma, and U-87 MG, a human glioblastoma cell line, were obtained through the American Type Tissue Collection (Rockville, MD). U-87 MG cells express wild-type p53 (p53wt), whereas U-373 MG cells express a mutated p53 (p53mut) gene product (16, 17). Human epidermoid carcinoma A 431 cells were also obtained from American Type Tissue Collection. The cell lines were tested for *Mycoplasma* contamination monthly, and only negative cells were used for experimentation.

Protease and phosphatase inhibitors and other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). All electrophoresis reagents were from Bio-Rad (Hercules, CA). The MEM- α (MEM Alpha) and the penicillin-streptomycin antibiotics were from Life Technologies, Inc. (Rockville, MD), and FBS was from Intergen (Purchase, NY).

The following immunological reagents from Neo Markers (Fremont, CA) were used: the mAb cocktail (Ab14) reacting with both the COOH- and the NH₂-terminal domains of EGFR, the anti-ErbB2 mAb cocktail (Ab10), reacting with both the extracellular and the cytoplasmic domains of ErbB2, and the anti-ErbB3 mAb (Ab7) and anti-ErbB4 mAb (Ab4) that react with the cytoplasmic domains of ErbB3 and ErbB4, respectively. Other antibodies were: anti-EGFR mAb from Transduction Laboratories (PharMingen/Transduction Laboratories, Los

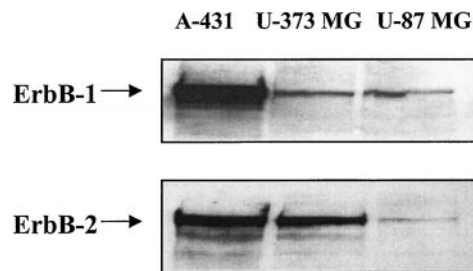


Fig. 1 Western blot analysis of the expression levels of EGFR and ErbB-2 in U-87 MG and U-373 MG cells compared with the squamous carcinoma cell line A-431. Cell lysates for each cell line were size fractionated by SDS-PAGE and immunoblotted with mAb specific for EGFR and ErbB-2. Protein standard assays were performed to control for equal loading of protein.

Angeles, CA), the anti-EGFRvIII mAb reacting with the NH₂-terminal truncation of EGFR, DH8.3 (AbCam Limited, Cambridge, United Kingdom), the immunoprecipitating anti-EGFR mAb, Ab5, the anti-phospho-Tyr mAb, Ab2, and peroxidase-conjugated goat antimouse Ab (Oncogene Science, Cambridge, MA). Protein A agarose was from Bio-Rad (Hercules, CA).

Mice and Tumors. Athymic female NCr-nu/nu nude mice were obtained from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). Animals used in this study were maintained under specific-pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. Solitary tumors were produced by s.c. inoculation of 10^7 cells into the right hind leg of 2-to-3-month-old mice. Tumor cell suspensions were prepared from U-87 MG cells grown as monolayers *in vitro*. Experiments were initiated when the tumors had reached a size of 8–10 mm in diameter.

Recombinant Ads and *in Vitro* Transduction Conditions. Replication-incompetent Ad was produced in bacteria as previously described (18, 19). The mutant EGFR-CD533 cDNA (20), lacking the 533 COOH-terminal amino acids, was kindly provided by A. Ullrich (Max-Planck-Institut fuer Biochemie, Martinsried, Germany). AdLacZ was used as control virus (19, 21). The transduction rate in AdLacZ-transduced cells expressing β -gal was determined by X-gal staining after 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside incubation for 24 h as described previously (21). To identify *lacZ* gene-negative cells more accurately, safranin O was used for counterstaining (22). Ad-EGFR-CD533 or AdLacZ was produced in 293 cells as described and purified by double CsCl gradient centrifugation followed by dialysis against 13% glycerol in PBS (19, 21). Virus was frozen in liquid nitrogen and stored at -70°C until further use. Titration by standard plaque assay indicated typical titers of 2×10^{11} pfu/ml.

Transduction of the cell lines with Ad-EGFR-CD533 or AdLacZ was carried out by diluting viral stocks with PBS, adding adenoviral preparations to cell monolayers (60-mm dish) and placing dishes on a rocker with gentle continuous agitation in a tissue culture incubator for 4 h at 37°C followed by medium

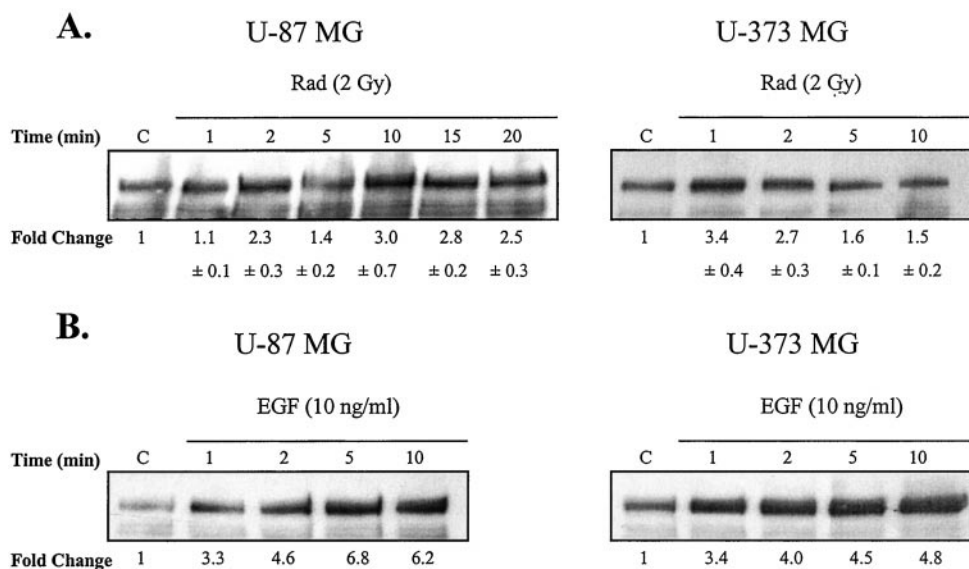


Fig. 2 Rad-induced (A) and EGF-induced (B) EGFR Tyr-P at specified times after Rad exposure of 2 Gy or 10 ng/ml of EGF in U-87 MG and U-373 MG cells. Cell lysates were immunoprecipitated with anti-EGFR mAb and size fractionated by SDS-PAGE; Western blots were reacted with anti-phospho-Tyr mAb, and the reaction was quantified by chemiluminescence and densitometric scanning. The corresponding values of relative phospho-Tyr levels represent increases over the negative control (Lane C). Values in A, representative of three independent experiments and are given as mean \pm SD.

change. The MOI (pfu/cell) was optimized for maximum transduction and minimum cell toxicity.

Cell Treatments and *in Vitro* Irradiation. U-87 MG and U-373 MG cells were seeded at 1.9×10^5 (60-mm dish)/ 8.0×10^5 (100-mm dish) or 1.5×10^5 (60-mm dish)/ 4×10^5 (100-mm dish), respectively, and cultured for a total of 4–5 days in MEM Alpha containing 10% FBS (MEM Alpha/10FBS) and penicillin/streptomycin. For clonogenic survival analyses, cells were transduced on day 3 with Ad-EGFR-CD533 or AdLacZ, or was left untransduced in 1 ml of medium (60-mm dish). For immunochemical analyses after irradiation or EGF treatments, medium was replaced by low-serum MEM Alpha/0.5FBS for 16 h prior to irradiation or EGF treatment. In all of the Rad experiments, cells were irradiated at a dose rate of 1.8 Gy/min using a ^{60}Co source. Cells were maintained at 37°C throughout the experiment except for irradiation at 20°C. For time course experiments, cells were irradiated with a single dose of 2 Gy and incubated for the times specified. Thereafter, medium was removed and cells were washed once in ice-cold PBS, immediately frozen on dry ice, and stored at -70°C until further processing.

EGFR Tyr-P and Western Blot Analysis. Immunoprecipitations and Western blotting were performed as described previously (9); briefly, frozen cells were lysed in ice-cold lysis buffer (consisting of 25 mM Tris, 50 mM β -glycerophosphate, pH 7.4, 5% (v/v) glycerol, 1.5 mM EGTA, 0.5 mM EDTA, 1% Triton, X-100, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 15 $\mu\text{g/ml}$ aprotinin, 15 $\mu\text{g/ml}$ leupeptin, 1 mM benzamide, and 100 $\mu\text{g/ml}$ PMSF). Cell lysis was facilitated by repeated passage through a 20-gauge needle, and insoluble material was removed by centrifugation at $14,000 \times g$ for 10 min. For time course experiments, equivalent amounts of protein, quantified by the Bradford Protein Standard Assay (Bio-Rad, Hercules, CA) were reacted with Ab5 at 4°C for 90 min followed by incubation with protein A-agarose for 45 min. Agarose was recovered by centrifugation, and immunoprecipitates were washed once in lysis buffer and twice in PBS.

Proteins were size-fractionated in 6% SDS-polyacrylamide gels, transferred electrophoretically onto nitrocellulose membranes (Bio-Rad, Hercules, CA), and probed with anti-phospho-Tyr mAb Ab2 (EGFR Tyr-P studies) or anti-EGFR mAb (EGFR-protein studies) followed by incubation with peroxidase-conjugated secondary Ab. After developing with CDP-Star (Tropix, Inc., Bedford, MA), autoradiograms were quantified using Sigma Scan software (Jandel Scientific, San Rafael, CA; Ref. 9). Ab14 was used for Western blotting of EGFR, Ab10 for ErbB2, and Ab7 and Ab4 for ErbB3 and ErbB4, respectively.

Colony Formation Assay. Cells were incubated on day 3 with either Ad-EGFR-CD533 or AdLacZ or were left untransduced in 1 ml of medium (60-mm dish) for 4 h followed by medium change. After 48 h, cells were irradiated with single doses of 2, 4, and 8 Gy and, after incubation for an additional 24 h, were harvested and plated for clonogenic survival. For repeated Rad exposure experiments, cells were exposed once daily to 2 Gy for 3 consecutive days without medium change. The number of cells was adjusted to generate 50–300 colonies per dish at each Rad dose and plated into 4-well 60-mm culture dishes. Cells were incubated at 37°C with 5% CO_2 for 12–14 days, stained with crystal violet, and colonies containing ≥ 50 cells were counted to determine surviving fraction (23).

Ad Infusion in U-87 MG Tumors and *in Vivo* Irradiation. At tumor diameters of 8–10 mm, AdLacZ (control) or Ad-EGFR-CD533 was delivered intratumorally as follows. Six 30-gauge needles were positioned as two sets of three needles in a triangular arrangement in opposing directions and penetrated to $\sim 60\%$ of the diameter of the tumor with appropriate spacing between the needles to allow for maximal Ad distribution. In addition, each needle was retracted 1 mm every 10 min during the infusion. A Bee Hive Controller and a Baby Bee Syringe Pump (Bioanalytical Systems, Inc. West Lafayette, Indiana) were used for all Ad infusions. All of the infusions were performed on fully anesthetized mice with a total volume of 20 μl per needle containing 1×10^9 pfu of AdLacZ or Ad-EGFR-

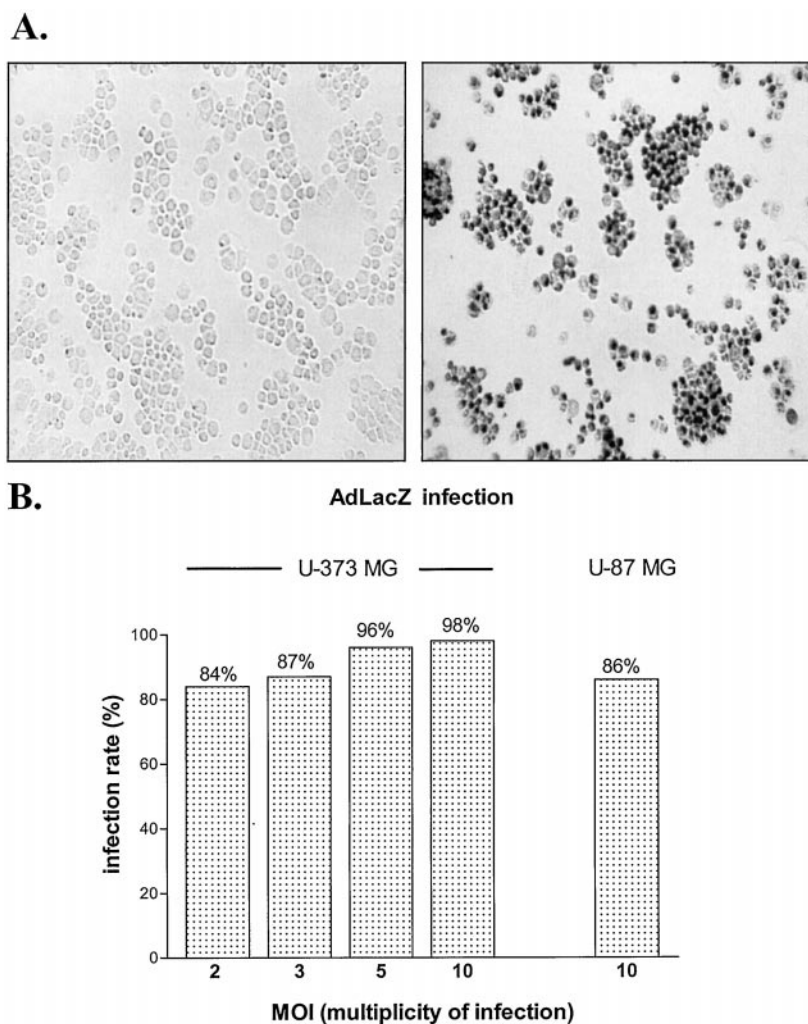


Fig. 3 A, nuclear X-gal staining for β -gal expression 48 h after transduction with AdLacZ (10 MOI) in U-87 MG cells at the time when Rad was performed in Rad experiments (as described in "Materials and Methods"). The nuclear staining of X-gal detects transduced cells with a blue color. Safranin O, used as counterstaining, stains cytoplasm and nuclei light red. The overall transduction rate was estimated as 86–89%. *Left panel*, X-gal staining and safranin O counterstaining for untransduced cells. *B*, U-373 MG cells were transduced at increasing MOI with LacZ expression recombinant Ad (AdLacZ) to determine the efficiency of transduction. The percentage of transduction was determined by counting 500 cells at each level of transduction and verifying the number of stained cells versus unstained cells. For additional experimental settings, 3 MOI were chosen for U-373 MG cells and 10 MOI for U-87 MG cells, respectively, with an overall transduction rate >85%.

CD533 using a PBS vehicle. The flow rate was 0.5 μ l/min. At 72 h postinfusion, mice were immobilized in a plastic box, and animals were centered in an 18 \times 18-cm field. A single 3-Gy dose of γ Rad was delivered whole body on 3 consecutive days at a dose rate of 2 Gy/min using a ^{60}Co source. Control tumors infused with AdLacZ or Ad-EGFR-CD533 were handled under mock-Rad conditions.

Measures of Tumor Radiosensitization. An *ex vivo* colony formation assay was performed to assess radiosensitization *in vivo*. Twenty-four h after irradiation, mice were killed, and tumors were excised and digested to single-cell suspension in 1.5 ml of collagenase (280 units/ml) and 1.5 ml of an enzyme cocktail [DNase (30.58 units/ml), Pronase (225 units/ml), and collagenase (3280 units/ml)], contained in 15 ml of MEM Alpha/10FBS plus antibiotics. After 2 to 3 h of digestion, single cells were filtered through a 40- μ m mesh filter, washed twice with media and counted. The cells were then plated for colony formation assay as described above.

Statistical Analysis. The Student *t* test was applied to establish statistical significance.

RESULTS

Characterization of EGFR and ErbB2 Protein Expression.

To define the expression levels of EGFR and ErbB2 in the two glioma cell lines, we confirmed surface receptor expression for each of the two cell lines relative to the A-431 cells using mAbs for EGFR and ErbB2. Immunoblotting experiments with Ab14 against EGFR revealed a single M_r 170,000 band in A-431, U-373 MG, and U-87 MG cell lysates (Fig. 1). However, the EGFR expression levels in the two glioma cell lines were substantially lower than in A-431 cells. No additional M_r 145,000–155,000 band, representing the receptor with a truncated COOH-terminal domain (EGFRvIII) was evident. Western blotting with Ab10 showed similar ErbB2 protein levels of the M_r 185 protein in A-431 and U-373 MG cells, whereas the ErbB2 expression level in U-87 MG cells was lower (Fig. 1). No ErbB3 or ErbB4 and no EGFRvIII bands were found in U-87 MG and U-373 MG cell lysates after immunoblotting with Ab7, Ab4, and anti-EGFRvIII respectively (data not shown). The results demonstrate that both glioma cell lines express significant levels of EGFR and ErbB2.

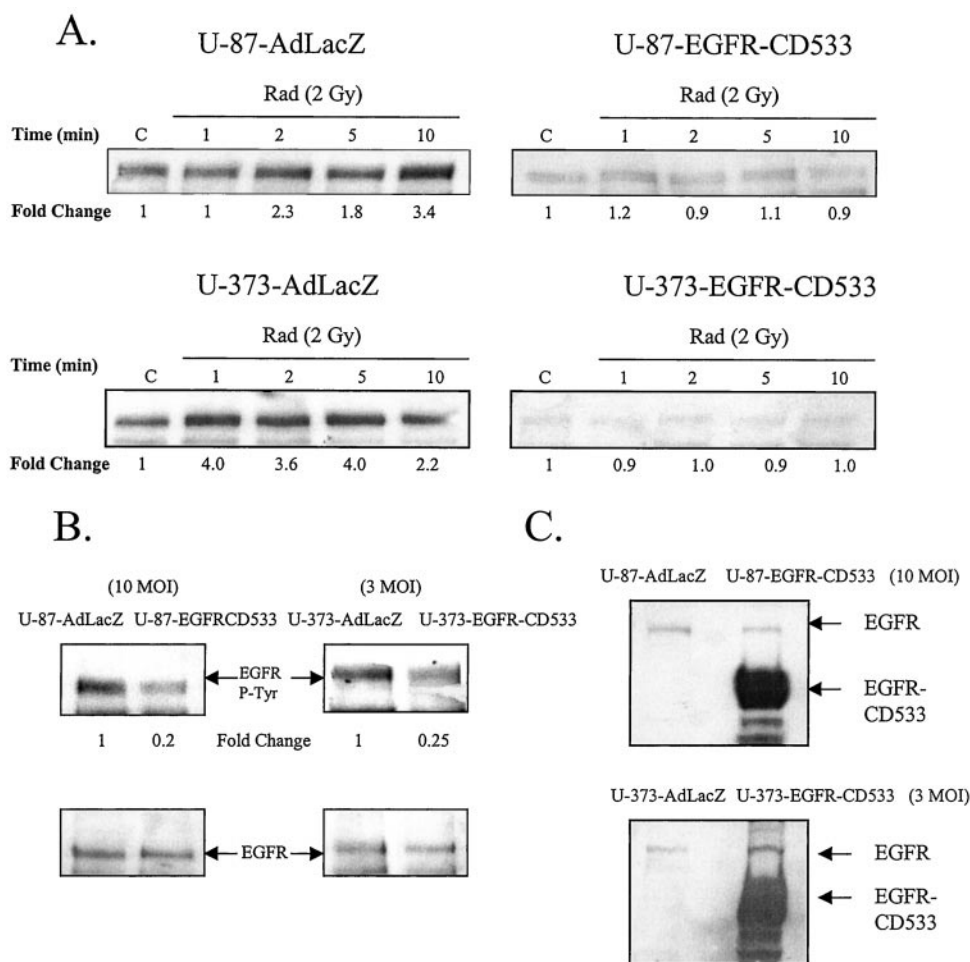


Fig. 4 A, Rad-induced EGFR Tyr-P is blocked by EGFR-CD533 expression. U-87 MG and U-373 MG cells were transduced with either control AdLacZ or Ad-EGFR-CD533 with a MOI of 10 or 3, respectively, and, 48 h later, were exposed to 2 Gy Rad. Cell lysates were immunoprecipitated with anti-EGFR mAbs and size fractionated by SDS-PAGE; Western blots were reacted with anti-phospho-Tyr mAb, and the reaction was quantified by chemiluminescence and densitometric scanning. B, the decrease in relative basal EGFR Tyr-P levels in Ad-EGFR-CD533-transduced cells over the control AdLacZ-transduced cells for both cell lines (*top lanes*). *Bottom lanes*, in parallel, cell lysates from the samples (*top lanes*) immunoprecipitated with anti-EGFR mAb, size fractionated by SDS-PAGE, and immunoblotted with a COOH-terminal binding anti-EGFR mAb to ensure equal EGFR protein loading. C, Western blot analysis of the expression levels of EGFR and EGFR-CD533 at the time, when Rad was performed in A. Cell lysates were size fractionated by SDS-PAGE and immunoblotted with a mAb cocktail reacting with both COOH- and NH₂-terminal domains of EGFR.

Rad-induced Activation of EGFR Tyr-P in U-87 MG and U-373 MG Cell Lines. We have previously demonstrated that Rad in the dose range of 0.5–5 Gy, similar to EGF stimulates EGFR Tyr-P in MCF 7, MDA-MB-231, and A-431 cells (10). To test these responses in the malignant glioma cells, U-87 MG and U-373 MG, EGFR activation after Rad and EGF exposure was quantified by relative levels of EGFR Tyr-P using Western analysis (Fig. 2). A 2-Gy Rad exposure resulted in maximum stimulation of EGFR activation in U-373 MG cells within 1 min, whereas U-87 MG cells showed a prolonged response with a maximum EGFR activation at 10 min (Fig. 2A). In three independent experiments, means of 3.0 (± 0.7)-fold (U-87 MG; 95% confidence interval, 1.86–3.18; $P < 0.005$) to 3.4 (± 0.4)-fold (U-373 MG; 95% confidence interval, 1.84–3.28; $P < 0.001$) increase relative to the controls were observed, which were responses in line with those previously found for human carcinoma cells (10, 14). EGF exposure led to a prolonged increase of EGFR Tyr-P in both of the cell lines (Fig. 2B).

Transduction of U-87 MG and U-373 MG Malignant Glioma Cells *in Vitro*. The overexpression of EGFR-CD533 was studied through a genetic approach of transducing cells with Ad-EGFR-CD533. To optimize the MOI for maximum

transduction with minimum cell toxicity in U-87 MG and U-373 MG cells, we evaluated different MOI between 3 and 30 for the transduction of cells with AdLacZ and Ad-EGFR-CD533. We examined the transduction rate of AdLacZ after X-gal staining (Fig. 3A), the expression levels of EGFR-CD533 by Western analysis and the toxicity by colony formation assay. We established that 10 MOI for U-87 MG and 3 MOI for U-373 MG cells produced 85–90% transduction efficiencies (Fig. 3B). Under these conditions, the survival rates by colony formation were 96 and 80% for U-87 MG and U-373 MG cells, respectively, relative to untransduced controls (data not shown). Overall, U-373 MG cells demonstrated a greater sensitivity to adenoviral transduction than did U-87 MG cells (Fig. 3B). The expression level of the newly expressed EGFR-CD533 was detectable 24 h after the transduction and approached maximum values by 48 h, as previously described (Ref. 13; data not shown).

Inhibition of Rad-induced Activation of EGFR Tyr-P by Overexpression of EGFR-CD533 via Ad-mediated Gene Transfer. Previous studies had established that expression of EGFR-CD533 does not affect the expression levels of EGFR but disrupts wild-type EGFR function and autophosphorylation through protein interaction (13). Rad-induced activation of

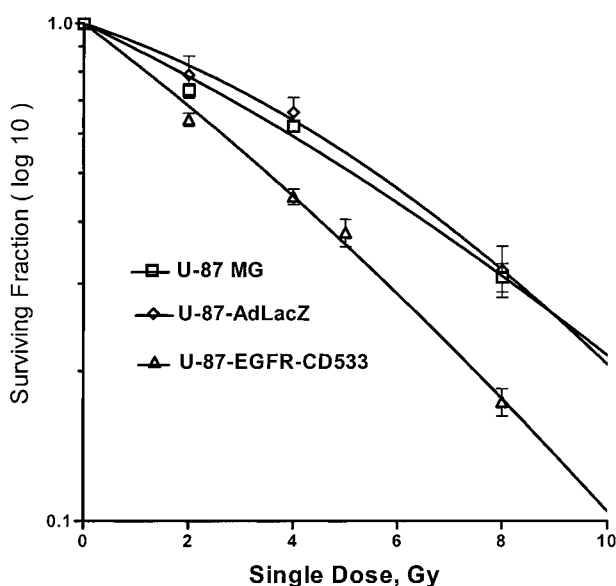


Fig. 5 Clonogenic survival after varying doses of single Rad exposure. Cells were either transduced with AdLacZ or Ad-EGFR-CD533 with a MOI of 10 for U87 or left untransduced and 48 h later were irradiated followed by a further incubation for 24 h at 37°C with 5% CO₂ before trypsinization and plating for clonogenic survival. After 12–14 days incubation, colonies were stained, and those with ≥ 50 cells were counted to determine the surviving fraction. The log survival was formed to the number of cells plated, after correcting for plating efficiency. Error bars, the mean \pm SEM in three experiments.

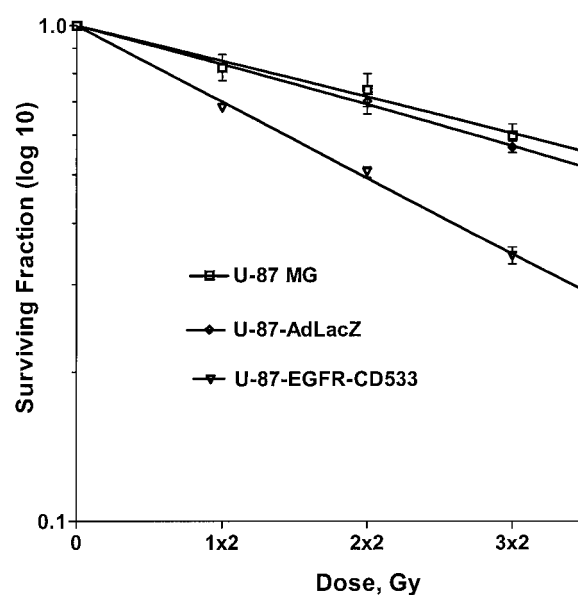


Fig. 6 Clonogenic survival after repeated Rad exposures of 2 Gy for 3 consecutive days. Cells were treated as described in Fig. 5 legend and 48 h later, were irradiated with a single dose of 2 Gy followed by incubation for 24 h before the next irradiation with 2 Gy. Rad was performed three times. Twenty-four h after each Rad exposure, cells were harvested and plated for clonogenic survival (Fig. 5 legend and “Materials and Methods”). Error bars, the mean \pm SEM in three experiments.

EGFR in U-87 MG and U-373 MG cells was inhibited through overexpression of EGFR-CD533 using the Ad-EGFR-CD533 and an AdLacZ control virus to assess the effects of vector alone on EGFR Tyr-P. The results shown in Fig. 4 demonstrate that AdLacZ did not affect the Rad-induced activation of EGFR in U-87 MG and U-373 MG cells. This EGFR activation was completely inhibited in U-87 MG and U-373 MG cells under conditions of EGFR-CD533 overexpression (Fig. 4A, *right lanes*). The basal Tyr-phosphorylation levels of EGFR were reduced to 12–20% and 4–25% (range in three independent experiments) in U-87 MG and U-373 MG cells, respectively, after transduction with Ad-EGFR-CD533 relative to AdLacZ controls (Fig. 4B, *top lanes*), without effecting basal EGFR-protein levels (Fig. 4B, *bottom lanes*). Fig. 4C illustrates the expression levels of EGFR-CD533 compared with EGFR at the time of Rad exposure 48 h after transduction. The expression level of the newly expressed EGFR-CD533 was substantially higher than the expression of the endogenous wild-type EGFR. This data supports previously published data on stably transfected MDA-MB-231 mammary carcinoma cells (13), demonstrating that the expression of EGFR-CD533 almost completely inhibits EGFR function.

In Vitro Radiosensitivity of U-87 MG and U-373 MG Cells under Conditions of EGFR-CD533 Expression. To assess the effect of inhibition of EGFR function through overexpression of EGFR-CD533 on the radiosensitivity of malignant glioma cells, we examined the single dose-response colony formation assay. As shown in Fig. 5, U-87 MG cells transduced with Ad-EGFR-CD533 were more radiosensitive over a range

of Rad doses compared with the AdLacZ control or untransduced cells. The D_{37} in the U-87-EGFR-CD533 cells was 1.4- to 1.5-fold lower relative to transduction with AdLacZ or untransduced U-87 MG cells. This effect was amplified in repeated Rad exposure experiments, 3×2 Gy, yielding a D_{37} ratio of 1.8–2.0 in comparison with controls (Fig. 6). This reduced clonogenic survival for cells expressing EGFR-CD533 was similar in the U-373 MG cells with a 38% survival reduction after a single dose of 4 Gy ($P < 0.0001$; Fig. 7).

In Vivo Radiosensitization in U-87 MG Tumor Xenografts after Ad-EGFR-CD533 Infusion. To determine the effect of EGFR-CD533 on tumor radiosensitization, U-87 MG tumor xenografts measuring 8–10 mm in diameter were infused *in vivo* with AdLacZ or Ad-EGFR-CD533 as described in “Material and Methods.” This technique routinely yielded transduction efficiencies of 59–65% (data not shown), as determined by X-gal staining of single cells, derived from tumor digests 3 days after AdLacZ infusion. Irradiation was performed 3 days after Ad infusion as described in “Material and Methods.” In this study, three fractions of 3 Gy were used based on the *in vitro* studies showing enhanced radiosensitization with Ad-EGFR-CD533 transduction after repeated Rad exposures (Fig. 6). Twenty-four h postirradiation, tumors were digested to single-cell suspension and *ex vivo* clonogenic survival was the treatment end point. The results presented in Fig. 8 show that the treatment with Ad-EGFR-CD533 and Rad resulted in a 44% survival reduction relative to the control treatment with AdLacZ and Rad (10.4 *versus* 18.5% survival; dose enhancement ratio, 1.8; $P < 0.001$). The plating efficiencies of tumor cells from

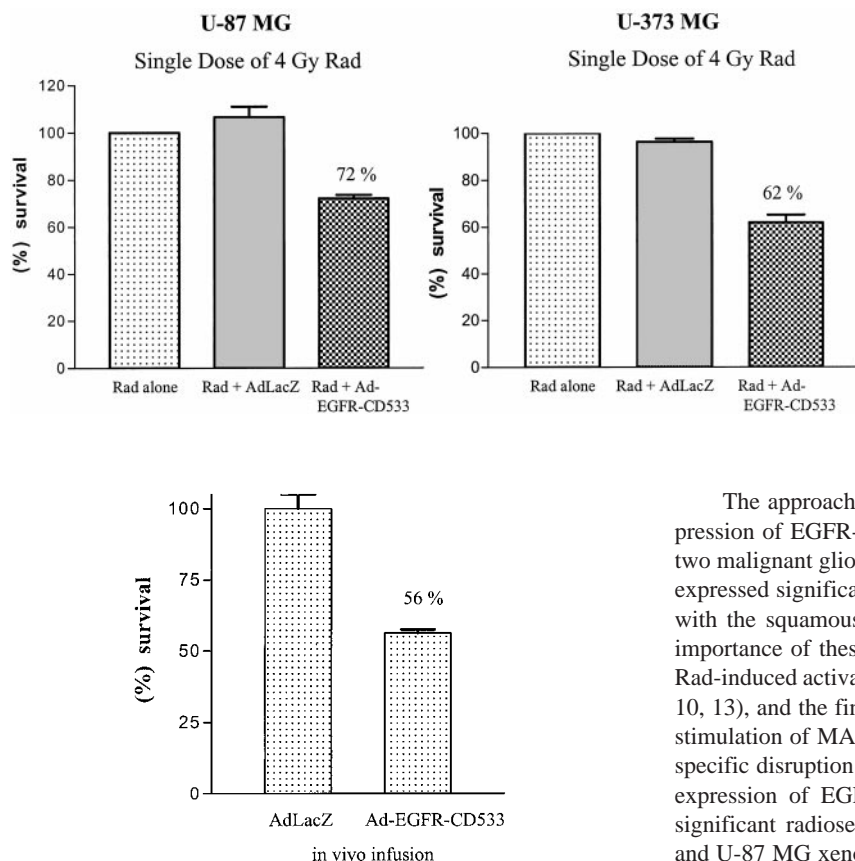


Fig. 8 Radiosensitization of U-87 MG tumor xenografts after infusion of Ad-EGFR-CD533. U-87 MG tumor xenografts with maximum diameters of 8–10 mm were treated with recombinant AdLacZ or Ad-EGFR-CD533 delivered intratumorally as described in “Materials and Methods.” At 72 h after infusion, animals were irradiated with 3 Gy/day for 3 days at a dose rate of 2Gy/min using a ^{60}Co source. At 24 h after Rad, tumors were excised, digested to single-cell suspension (as described in “Materials and Methods”), and cells were plated for clonogenic survival. Survival rate for AdLacZ-infused tumor cells was set as 100% after correcting for plating efficiency (Fig. 5 legend and “Materials and Methods”). Results, data from two independent experiments performed in quadruplicate; error bars, \pm SEM. Means are significantly different (t , 10.84; $P < 0.0001$). The dose enhancement ratio reaches 1.8.

AdLacZ- and Ad-EGFR-CD533-infused tumors were similar (6.79 versus 6.14%; $P > 0.5$).

DISCUSSION

Our experimental results will be discussed in the context of using overexpression of EGFR-CD533 for the radiosensitization of human malignant glioma cells. This follows our previous findings that Rad at doses between 0.5 and 5 Gy activates EGFR in squamous and mammary carcinoma cell lines (9, 10, 14) and mediates a cytoprotective proliferative response counteracting the toxic effects of Rad (13). Considering the extreme radioresistance of malignant gliomas (24), we thought that accelerated proliferation may be one contributing mechanism to this resistance, in part supported by encouraging improvements in tumor control rates using an accelerated dose escalation radiotherapy regimen (25).

Fig. 7 Clonogenic survival rate after a single Rad exposure of 4 Gy in U-87 MG (A) and U-373 MG cells (B), transduced with either AdLacZ or Ad-EGFR-CD533 (in relation to untransduced cells). Survival rate for untransduced cells was set as 100% after correcting for plating efficiency (Fig. 5 legend and “Materials and Methods”). The additive cell-killing effect for the group of Ad-EGFR-CD533 transduced cells compared with control transduced (AdLacZ) or untransduced cells was nearly the same in both cell lines ($P < 0.0001$).

The approach of glioma cell radiosensitization by overexpression of EGFR-CD533 was justified by our finding that the two malignant glioma cell lines, U-87 MG and U-373 MG, both expressed significant levels of EGFR and ErbB2 in comparison with the squamous carcinoma cell line A-431. The functional importance of these ErbB receptors was demonstrated by their Rad-induced activation similar to that in other cell systems (6, 9, 10, 13), and the finding that EGFR activation was linked to the stimulation of MAPK (11).⁴ In this report, we demonstrate that specific disruption of EGFR function under conditions of overexpression of EGFR-CD533 after Ad transduction results in significant radiosensitization of both human glioma cell lines and U-87 MG xenografts. Importantly, the radiosensitization of glioma cells was seen after single-dose exposures, resulting in an enhancement ratio of 1.5, and an even greater value of 2.0, after three consecutive 2-Gy exposures.

Transduction of both U-87 MG and U-373 MG glioma cells with Ad-EGFR-CD533 results in massive overexpression of the DN receptor (Fig. 4C). This suggests that the mechanism of action of EGFR-CD533 is at the protein level similar to that previously described (11, 13) because of preferred dimerization of EGFR-CD533 with EGFR wild-type and other ErbB receptors, thus seriously compromising their ability for intracellular signaling. The functional implications of disrupting the Rad-induced activation of EGFR with EGFR-CD533 in glioma cell responses are presently examined in more detail. According to our previous findings in other human carcinoma cell lines, signaling along the MAPK and c-jun NH₂-terminal kinase are important candidate pathways in cellular Rad response (11) and have already been partially verified in glioma cells.⁴ In addition, we will extend our radiobiological studies to the EGFR-mediated modulation of PI-3-kinase, which also plays a critical role in cellular Rad responses (26, 27).⁵

The mechanisms underlying radiosensitization are still incompletely understood. The linkage between Rad-induced EGFR activation and proliferation represents one potential

⁴ G. Lammering, T. H. Hewit, P. Dent, and R. K. Schmidt-Ullrich, unpublished observations.

⁵ J. Contessa, J. L. Hamton, G. Lammering, P. Dent, and R. K. Schmidt-Ullrich, unpublished observations.

mode, which is completely eliminated by the overexpression of EGFR-CD533. This contribution of Rad-induced proliferation to radiosensitization is most directly demonstrated by repeated Rad exposure experiments (10, 11, 13, 14). Another contributing factor may act through the inhibition of MAPK cascade and other cytoplasmic protein kinases, which control many transcription processes associated with cell proliferation and repair, which are indirectly linked to enhanced biosynthetic processes (28–30). The cellular Rad responses are additionally facilitated by altered control of cell cycle checkpoints. For example, delays in G₁-S phase and G₂-M transitions may be interpreted as the tendency of the cells to provide improved conditions or additional time for DNA damage repair (27, 31). These conclusions are increasingly supported by direct experimental evidence provided by the links between EGFR/MAPK activation and the induction of the cyclin-dependent kinase inhibitor p21^{WAF/CIP1} (27, 32).

This radiosensitizing effect is at variance with one of our previous reports on stably transfected MDA-TR15-EGFR-CD533 mammary carcinoma cells in which no radiosensitization after single Rad exposures was observed (13). This radiosensitization occurred only under conditions of 24-h delayed plating. Previously negative radiosensitization experiments on cells irradiated in suspension immediately after trypsinization further underline the importance of intact EGFR/ErbB RTK function in the Rad response of carcinoma and glioma cells.

Currently, different approaches of inhibiting EGFR function for radiosensitization are being investigated. Tyrphostin and other Tyr kinase inhibitors directed at individual or all (10, 33, 34) ErbB receptors have shown effectiveness in carcinoma cell radiosensitization. Another major area of research concentrates on mAbs against EGFR, C225 being the best characterized and most widely applied one. This C225 mAb has been shown to radiosensitize human and murine carcinoma cells both *in vitro* and *in vivo* after single and repeated Rad exposures (35, 36). Relative to the pharmacological and immunotherapeutic approaches, the genetic therapy strategy demonstrated here has the advantage that the overexpression of DN-EGFR-CD533 likely affects all ErbB receptors irrespective of their varied expression levels by different carcinoma cells,⁶ including receptors with truncated NH₂-terminal domains, *e.g.*, EGFRvIII (37, 38), thus lacking GF and mAb binding moieties.

In summary, this report demonstrates that human glioma cells express significant but varied levels of EGFR and ErbB2. The Rad-induced activation of these receptors was almost completely inhibited by the overexpression of EGFR-CD533 after infection of cells with the adenoviral vector. The overexpression of EGFR-CD533 resulted in marked radiosensitization *in vitro* and *in vivo* as demonstrated by significantly enhanced cytotoxicity after single or repeated Rad exposures. The results presented strongly suggest that overexpression of EGFR-CD533 may be developed into a genetic therapy approach of radiosensitizing human gliomas in which EGFR and other ErbB RTKs are part of the autocrine growth-regulatory circuit.

⁶ R. K. Schmidt-Ullrich, G. Bouwers, and G. Lamering, unpublished observations.

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Clin Cancer Res 2001;7:682-690.

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