The Inducible Expression of Dominant-Negative Epidermal Growth Factor Receptor-CD533 Results in Radiosensitization of Human Mammary Carcinoma Cells

Joseph N. Contessa, Dean B. Reardon, Daniel Todd, Paul Dent, Ross B. Mikkelsen, Kristoffer Valerie, Geoffrey D. Bowers, and Rupert K. Schmidt-Ullrich


ABSTRACT

Ionizing radiation activates the epidermal growth factor receptor (EGFR) and downstream signaling involving the cytoprotective mitogen-activated protein kinase (MAPK) pathway. In our effort to investigate the role of EGFR in cellular responses to radiation, we generated mammary carcinoma cell clones, MCF-TR5-EGFR-CD533 and MDA-TR15-EGFR-CD533, that inducibly express EGFR-CD533, a truncated EGFR mutant lacking mitogenic and transformation activity. EGFR-CD533 expression inhibits radiation- and EGF-induced EGFR autophosphorylation and MAPK activation and, therefore, functions as a dominant-negative mutant without blocking the expression of EGFR or erbB-2, another member of the erbB receptor Tyr kinase family. Expression of EGFR-CD533 only minimally inhibited cell growth and did not alter radiosensitivity to single radiation exposures. However, repeated 2 Gy radiation exposures of cells, under conditions of EGFR-CD533 expression, essentially abolished their ability for subsequent cell growth. These results identify the inhibition of EGFR function through genetic manipulation as a potential therapeutic maneuver. The concept of such an intervention would be the radiosensitization of cells by counteracting a radiation-induced cytoprotective proliferation response.

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INTRODUCTION

Radiation-induced proliferation after relatively low, therapeutically applied doses has been described in experimental systems (1) and has been indirectly derived from tumor control analyses of clinical experiences (2, 3). These results, suggesting preferential accelerated proliferation during the latter part of a typical radiotherapy course, support the conclusion that this response in tumors adversely affects treatment outcomes (2–4).

Studies from our laboratory on human MCCs and SCCs have identified a possible mechanism of radiation-induced proliferation by demonstrating that ionizing radiation in the 0.5–5 Gy dose range activates the EGFR in these cells to a similar extent as mitogenic concentrations of EGF (5, 6). We have further demonstrated that the radiation-induced activation of EGFR is linked to a proliferation response after single and repeated radiation exposures (7) and is transmitted by the MAPK cascade (6, 8, 9). The cytoprotective nature of the MAPK response is compatible with the notion that radiation-induced proliferation represents an adaptive response counteracting the toxic effects of ionizing radiation. Data on the radiosensitizing effect of a MAPK inhibitor (8) and the finding that neutralizing antibody against EGFR can sensitize A431 cells to radiation (10) identify the disruption of EGFR function as a potential tool for intervention of cytoprotective radiation responses.

For studies on the role of EGFR in cellular radiation responses, we have used a genetic approach and generated stable constructs of human MCCs in which the function of EGFR can be modulated in a controlled fashion. In this report, we describe the effects of the inducible expression of the dominant-negative EGFR mutant, EGFR-CD533, lacking mitogenic and transformation activity (11), on radiation-induced activation of the EGFR and critical cytoprotective responses, such as MAPK activation. We also demonstrate that this genetic approach of disrupting EGFR function can result in a significant enhancement of radiation toxicity using a novel assay system that was designed to study the interactions between repeated radiation exposures and cell proliferation.

MATERIALS AND METHODS

Reagents. Unless specified otherwise, all reagents were from Sigma Chemical Co. (St. Louis, MO). The immunochemi-

3 The abbreviations used are: MCC, mammary carcinoma cell; SCC, squamous carcinoma cell; EGFR, epidermal growth factor receptor; EGFR-WT, 170 kD EGFR wild-type; EGFR-CD533, dominant negative M, 110,000 truncated EGFR; MAPK, mitogen-activated protein kinase; FBS, fetal bovine serum; Dox, doxycycline; mAb, monoclonal antibody; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.
ural reagents have been described (5, 7, 8), except for the following. A mAb cocktail reacting with both COOH- and NH$_2$-terminal domains of EGFR (Ab14; Neo Markers, Fremont, CA) was used for Western blotting of EGFR. mAb E12120 (Transduction Laboratories, Louisville, KY), which reacts specifically with autophosphorylated EGFR, was used for probing blots for activated EGFR. ErbB-2 was immunoprecipitated and blotted with rabbit polyclonal Ab sc-284 (Santa Cruz Biotechnology, Santa Cruz, CA). Radioisotopes were from Du Pont-New England Nuclear (Boston MA). Tetracycline-free FBS was from Clontech (Palo Alto, CA), and RPMI 1640 was from Life Technologies, Inc. (Gaithersburg, MD).

**Construction and Characterization of Cell Lines.** The parental MCC lines, MCF-7 and MDA-MB-231, have been described (6, 9). For optimal efficiencies, transfections of MCF-7 or MDA-MB-231 cells were performed by the calcium phosphate and electroporation methods, respectively (12). Stable clones expressing the tetracycline resistance transactivator were selected after transfection with pUHD172–1neo (provided by A. Ullrich, Max-Planck-Institut fuer Biochemie, Martinsried, Germany), cloned as a 1.75-kb XbaI fragment into pUHC10–3 containing the tet operator and the luciferase reporter gene (13). The pRK5HerNa8 plasmid encoding the EGFR cDNA, lacking the COOH-terminal 533 amino acids (Ref. 10; provided by A. Ullrich, Max-Planck-Institut fuer Biochemie, Martinsried, Germany), was cloned as a 1.75-kb XbaI fragment into pUHC10–3 containing the tet operator to yield the pUHC10–3–EGFR-CD533. To obtain EGFR-CD533-expressing cells, the MCF-7-TR5 and MDA-TR15 clones from the first level of transfections were cotransfected with this plasmid and pRSVHygro, 20.4 μg/10 cm dish, and selected for resistance to hygromycin at 200 μg/ml. Vector control cell lines, MDA-TR15-Luc27 and MCF-TR5-Luc24, were obtained by cotransfection with pUHC10–3 containing the luciferase reporter gene and pRSVHygro.

Induction of EGFR-CD533 was quantified at the level of mRNA and protein after 48 h induction by Dox at 1 μg/ml (13). The cytoplasmic mRNA levels in MCF-TR5-EGFR-CD533 cells were quantified by RNase protection, and Northern analysis was used for MDA-TR15-EGFR-CD533 cells using established procedures (14). The kinetics of EGFR-CD533 induction and its decay after Dox removal were monitored by metabolic labeling with [35S]Met as described in the text.

The possible effects of EGFR-CD533 expression on the protein levels of EGFR-WT and erbB-2 were examined by Western blotting of cell lysates or immune precipitates (6). Tyr autophosphorylation of EGFR was monitored as described previously (5, 6) with the following modifications. Dox at 1 μg/ml was added 48 h before radiation exposure and cell harvesting with a medium change to RPMI/0.5%FBS with or without Dox for the final 24 h. This medium was replaced by RPMI/0.5%FBS 2 h before treatment. Under these conditions, cell cultures were at 70–80% confluence, and experimental treatments were initiated, such as irradiation with 60Co at a dose rate of 1.7 Gy/min at 20°C or exposure to 2 ng/ml of EGF for 5 min. MAPK assays were carried out identically to procedures described for EGFR Tyr phosphorylation, followed by immune complex assays (8, 9).

Besides using single radiation dose response analyses (15), we have devised a scheme of repeated radiation exposures combined with a MTT cell growth assay (see Fig. 6; Refs. 6 and 7). This assay was specifically designed to investigate interactions between repeated radiation exposures and cell proliferation and was applied to demonstrate the effect of EGFR-CD533 expression on cell survival. MDA-TR15-EGFR-CD533 cells were seeded in RPMI/5%FBS at 1000 or 2000 cells/well in 96-well tissue culture plates for control and radiation experiments, respectively. After cell attachment overnight, 50% of the cultures were exposed to Dox at 1 μg/ml. Within 24 h of Dox exposure, drug-exposed and control cultures were irradiated once daily with 2 Gy for 5 consecutive days. Without additional feeding, cell growth was assessed on days 1, 4, and 7 after irradiation using the MTT assay (6, 7).

**RESULTS**

**Induction of EGFR-CD533 mRNA and Protein.** For our studies on the mechanisms and the modulation of radiation-induced cell proliferation, a dominant-negative EGFR mutant, EGFR-CD533, was chosen and expressed, for the first time, in human carcinoma cells (11). The dependence of autocrine growth-regulated carcinoma cells on EGFR-WT function also dictated the use of an inducible expression system because of potential toxic effects under conditions of constitutive expression. We have used the two plasmid tetracycline-on expression system (13). From the initial transfection with pUHD172–1neo, the MCF-TR5 and MDA-TR15 clones were chosen based on the extent of inducible EGFR-CD533 expression as quantified by the luciferase reporter plasmid, pUHD13–3. These cloned cell constructs were then cotransfected with pUHC10–3–EGFR-CD533 and pRSVHygro and yielded the stable clones MCF-TR5-EGFR-CD533 and MDA-TR15-EGFR-CD533.

The MCF-TR5-EGFR-CD533 cells produced a 5-fold increase in EGFR-CD533 mRNA levels after 48 h of Dox exposure (Fig. 1A). This translated into a greater than 20-fold induction of the M, 110,000 EGFR-CD533 truncated receptor. On the other hand, the MDA-TR15-EGFR-CD533 cells demonstrated a >30-fold increase in EGFR-CD533 mRNA levels (Fig. 1B) and a >50-fold induction at the protein level. Because of the higher EGFR-WT expression levels in MDA-MB-231 cells, we selected MDA-TR15-EGFR-CD533 cells for illustrating the responses described below.

**Dose Dependence and Kinetics of EGFR-CD533 Induction by Dox.** The induction and decay kinetics of EGFR-CD533 protein were examined. At 48 h exposure to Dox between 0.01 and 1.0 μg/ml, a half-maximal response was seen at 0.1 μg/ml, which is consistent with previous reports (Fig. 2A; Ref. 13). In studying the induction kinetics of EGFR-CD533, the fastest rate of induction at 1.0 μg/ml Dox measured by [35S]Met incorporation occurred between 8 and 24 h, at which time the EGFR-CD533 had been induced by >90% (Fig. 2B, upper panel). Thereafter, the rate leveled off substantially, and maximum induction was achieved by 48 h. The decay kinetics were measured after 48 h induction, followed by Dox removal. A 65% reduction of EGFR-CD533 was seen after 24 h, reaching 90% at 48 h (Fig. 2B, lower panel). The studies illustrated were obtained with MDA-TR15-EGFR-CD533 cells but were found to be identical to those for MCF-TR5-EGFR-CD533 cells (data...
against the NH2-terminal portion of the protein, a Western blot function of EGFR-WT. Using a mixture of mAbs (Ab14) of EGFR-WT.

It was important to examine whether the ex-

pendent experiments.

170,000 EGFR-WT was identified in cells lysates from [35 S]Met-la-

eld A431 cells. The results shown are representative of three inde-

sponses.

Effects of EGFR-CD533 expression on cell proliferation after repeated radiation exposures. To assess the possible effect of EGFR-CD533 expression on the radiosensitivity of MCCs, we performed single radiation dose-response clonogenic survival analyses between 1 and 8 Gy (15). Neither MCF-TR5-EGFR-CD533 nor MDA-TR15-EGFR-CD533 cells exhibited significant differences in radiosensitivity. The values of mean inactivation doses D (17) for the MDA-TR15-EGFR-CD533 cells with or without Dox exposure were 1.72 and 1.79, respectively, and for the MCF-TR5-CD533 cells, 1.69 and 1.54, respectively. Both data pairs were not significantly different.

We next designed the repeated radiation exposure experiments in an attempt to amplify potential interactions between radiation toxicity and the growth inhibitory effects of EGFR-

CD533 expression. Because of relatively low cell numbers, the MTT assay did not discern significant differences between the four combinatorial conditions of EGFR-CD533 expression and irradiation on day 1 of the 7-day growth assay (Fig. 6). Over 7 days, the unirradiated cell cultures grew at similar rates irrespective of EGFR-CD533 expression with a modest but significant growth retardation of the MDA-TR15-EGFR-CD533 cells under conditions of EGFR-CD533 expression (Fig. 6A).

In contrast, expression of EGFR-CD533 markedly affected the

Effects of EGFR-CD533 expression on MAPK responses to Radiation Exposure. We next examined the effects of EGFR-CD533 expression on MAPK activity because MAPK represents a critical link between EGFR activation and cellular proliferation responses induced by ionizing radiation (6). The responses of MAPK were examined >120 min for MDA-TR15-EGFR-CD533 cells after exposure to 2 Gy with and without prior exposure to Dox. Radiation induced an immediate, ~2-fold activation of MAPK, as demonstrated in Fig. 5A. This MAPK response was completely inhibited after exposure of EGFR-CD533. The responses were not due to the transfection or Dox exposure, because cells stably transfected with the luciferase control vector, in place of EGFR-CD533, demonstrated a radiation-induced MAPK response irrespective of Dox exposure (Fig. 5B). A similar radiation-induced MAPK response was observed with MCF-TR5-EGFR-CD533 cells (Fig. 5C). These results demonstrate that EGFR function is essential for radiation-induced activation of MAPK and suggest that disruption of this cytoprotective pathway may allow the modulation of cellular radiation responses.

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Fig. 1 Dox inducibility of EGFR-CD533 in MCF-TR5-EGFR-CD533 and MDA-TR15-EGFR-CD533 cells. mRNA was quantified for MCF-TR5-EGFR-CD533 cells by RNase protection assays (A) and by Northern analyses for MDA-TR15-EGFR-CD533 cells (B). The expression of the M, 110,000 EGFR-CD533 protein was determined by a [35 S]Met, 50 µCi/ml for 6 h, labeling 48 h after Dox exposure at 1 µg/ml, followed by immunoprecipitation and size-fractionation by SDS-PAGE. The M, 170,000 EGFR-WT was identified in cells lysates from [35 S]Met-la-

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sponses.

Effects of EGFR-CD533 on Expression and Activation of EGFR-WT. It was important to examine whether the expression of EGFR-CD533 affected the expression levels or the function of EGFR-WT. Using a mixture of mAbs (Ab14) against the NH2-terminal portion of the protein, a Western blot analysis of immunoprecipitated EGFR-WT and EGFR-CD533 indicated that even with a robust induction of EGFR-CD533, EGFR-WT protein expression levels remained essentially con-

stant, with a suggested minimal decline at 120 h (Fig. 3A).

Expression of other erbB family members of MDA-MB-231 cells (16), including erbB-2 shown in Fig. 3C, was also unaffected by EGFR-CD533 expression, except at later time points of induction. However, when a mAb specific for the autophos-

phorylated form of EGFR was used in a Western blot analysis of immunoprecipitated EGFR-WT and EGFR-CD533, there was a time-dependent marked effect on Ab reactivity (Fig. 3B); within 48 and 120 h of Dox exposure, the expression of phosphorylated EGFR-WT was reduced to 40 and <10%, respectively, of the control. These studies indicate that expression of EGFR-CD533 has only marginal effects on EGFR-WT expression but interferes with receptor activation.

Previously, we demonstrated that EGFR becomes rapidly activated as assessed by Tyr phosphorylation after radiation exposures in the 0.5–5 Gy dose range, and that this activation can be linked to a proliferation response in A431 SCCs (5–7). The results shown in Fig. 4A (left eight lanes) demonstrate that MDA-TR15-EGFR-CD533 cells responded to 2 Gy of radiation and exposure to 2 ng/ml of EGF with EGFR-WT activation in a similar way as had been shown for the parental MDA-MB-231 cells (5). In the absence of Dox, MDA-TR15-EGFR-CD533 cells showed a 3.8-fold increase in EGFR-WT Tyr phosphorylation within 1 min of radiation exposure, which decreased to baseline activity within 10 min. In comparison, 2 ng/ml of EGF induced a 9.8-fold activation that maximized at about 5 min. Importantly, these responses were completely ablated in MDA-

TR15-EGFR-CD533 cells under conditions of maximum EGFR-CD-533 expression (Fig. 4A, right eight lanes). The expression of the luciferase control vector did not affect the radiation-induced activation of EGFR-WT (Fig. 4B). These data demonstrate that the expression of EGFR-CD533 converted autocrine growth-regulated MCCs into functionally dominant-negative EGFR mutants.

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A. MCF-TR5-CD533

mRNA - + Dox

35S-Metabolic Labeling

EGFR CD533

- + Lysate

Dox

B. MDA-TR15-CD533

mRNA - + Dox

35S-Metabolic Labeling

EGFR CD533

- + Dox

Fig. 1 Dox inducibility of EGFR-CD533 in MCF-TR5-EGFR-CD533 and MDA-TR15-EGFR-CD533 cells. mRNA was quantified for MCF-TR5-EGFR-CD533 cells by RNase protection assays (A) and by Northern analyses for MDA-TR15-EGFR-CD533 cells (B). The expression of the M, 110,000 EGFR-CD533 protein was determined by a [35 S]Met, 50 µCi/ml for 6 h, labeling 48 h after Dox exposure at 1 µg/ml, followed by immunoprecipitation and size-fractionation by SDS-PAGE. The M, 170,000 EGFR-WT was identified in cells lysates from [35 S]Met-labeled A431 cells. The results shown are representative of three inde-

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The results shown in Fig. 4A (left eight lanes) demonstrate that
growth of MDA-TR15-EGFR-CD533 cells after five daily 2 Gy radiation exposures (Fig. 6B). Without the expression of EGFR-CD533, the irradiated cells resumed proliferation rates on days 4 and 7 that were similar to or even exceeded those of the unirradiated control cultures. In contrast, the cultures irradiated under conditions of EGFR-CD533 expression demonstrated significant growth retardation, which was reflected in significantly reduced cell numbers on days 4 and 7 (Fig. 6B). Therefore, on day 7 of the growth period, MDA-TR15-EGFR-CD533 cells expressing EGFR-CD533 during and after irradiation yielded a minimum of 4-fold lower cell numbers, demonstrating that the expression of EGFR-CD533 enhanced radiation toxicity to that same extent. These results illustrate that irradiation schedules may have to be modified, depending on the experimental condition applied, and demonstrate that EGFR-WT function is required for carcinoma cell proliferation responses after repeated radiation exposures.

**DISCUSSION**

Ionizing radiation at doses between 0.5 and 5 Gy has been shown to activate EGFR in human carcinoma cell lines and to mediate a proliferation response in A431 cells at mildly growth-inhibitory concentrations of EGF (6, 7). To explore novel therapeutic approaches, we have applied the genetic modulation of EGFR function because our previous work suggests that the radiation-induced EGFR activation initiates a cytoprotective response mediated through the MAPK pathway. The genetic approach used was to generate MCC lines, MCF-TR5-/MDA-TR15-EGFR-CD533, in which the dominant-negative EGFR-CD533 (11) was expressed under the control of a Dox-inducible promoter. This approach was essential because EGFR is part of
the autocrine growth regulatory system in the MCCs used. The critical role of radiation-induced EGFR activation in initiating downstream components of the proliferation signal transduction cascade was confirmed by our experimentation. Dox-induced expression of the EGFR-CD533 occurred within 24–48 h, ablated the radiation-induced activation of EGFR-WT as measured by Tyr phosphorylation, and therewith converting these cells into dominant-negative EGFR mutants. The importance of radiation-induced EGFR activation on downstream signaling was further elucidated by the inhibitory effects of EGFR-CD533 expression on MAPK activation and on cell proliferation after repeated 2 Gy radiation exposures resembling clinically applied irradiation.

That EGFR-CD533 behaves as a dominant-negative mutant is supported by the finding that the radiation- and EGF-induced EGFR-WT activation is almost completely inhibited after EGFR-CD533 expression (Fig. 4). This result is in agreement with earlier work in mouse NIH3T3 cells (11, 18); however, this effect had not been demonstrated previously in carcinoma cells in which EGFR represents a critical component of the autocrine growth regulatory system (11, 18). The finding that EGFR-CD533 has essentially no effect on the expression of EGFR-WT or ErbB-2, expressed at a significant level in MDA-MB-231 cells (Fig. 3; Ref. 16), is intriguing and is presently examined in more detail. Therefore, these data strongly suggest that the complete inhibition of EGFR-WT activation by excess amounts of EGFR-CD533 occurs through inhibition at the protein level, most likely through heterodimerization with EGFR-WT, an interaction that can be expected to prevent its autophosphorylation.

The functional implications of radiation-induced activation of EGFR-WT in cellular responses was further validated by examining MAPK as a critical component of the proliferation pathway, which has been shown to be activated by low doses of radiation and EGF in an EGFR-dependent fashion. The linkage between MAPK activation and a proliferation response after radiation exposures has been established previously in A431 SCCs using the EGFR kinase inhibitor AG1478 (6). The specificity of the EGFR-CD533 effect is unambiguous, and complete inhibition of MAPK activation after radiation exposure demonstrates that the expression of EGFR-CD533 can disrupt the MAPK signaling pathway.

EGFR-CD533 expression does not alter cellular radiosensitivity as determined in single radiation dose response analyses (15). However, the newly developed, repeated radiation exposure schedule under conditions of EGFR-CD533 expression, followed by a 7-day proliferation assay, showed a profound enhancement of radiation toxicity. These data demonstrate that disruption of an EGFR-WT-mediated cytoprotective response prevents recovery of cell proliferation after repeated radiation exposure. Extrapolation of this data to even more protracted radiation exposure schemes, as applied in clinical radiotherapy, suggests that disruption of the cytoprotective EGFR/MAPK signal transduction cascade holds promise of significantly enhancing the cytotoxic effects of ionizing radiation. The mechanisms underlying these responses downstream of MAPK require additional investigation. One possibility is that induction of the MAPK proliferation response enhances the cells’ biosynthetic machinery, including their ability to repair radiation damage. The other, possibly related to the former, may be based on recently established links between MAPK activation and the induction of the cyclin-dependent kinase inhibitor, p21WAF1/CIP1 (8, 19) which, through cell cycle arrest, may facilitate DNA damage repair. These processes would be inhibited in cells expressing EGFR-CD533, thus resulting in more severe DNA damage manifestation. In addition, modest retardation of cell growth upon expression of EGFR-CD533 during repeated radi-

![Figure 4](image-url)
ation exposures may be amplified and prevent cells from entering a phase of accelerated proliferation (1–4, 6, 7).

In summary, this report outlines, for autocrine growth-regulated carcinoma cells, a novel approach of genetic disruption of an EGFR-initiated cytoprotective response after irradiation. The demonstration of significantly enhanced cytotoxicity after repeated radiation exposures of cells expressing EGFR-CD533 was made possible by a specifically devised growth assay. These promising results of enhanced radiation toxicity strongly suggest that the genetic manipulations used can be exploited therapeutically. Such a therapeutic application could be accomplished through efficient transient transduction of carcinoma cells using recombinant adenovirus EGFR-CD533 or through low molecular weight specific inhibitors of critical components of the proliferation pathway, such as EGFR and MAPK.

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