Inverse Relationship between Epidermal Growth Factor Receptor Expression and Radiocurability of Murine Carcinomas

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ABSTRACT
The study investigated whether a relationship exists between the extent of epidermal growth factor receptor (EGFR) expression and in vivo radiocurability of murine tumors. EGFR expression was determined in nine carcinomas (four mammary carcinomas, designated MCa-4, MCA-29, MCA-35, and MCA-K; two squamous cell carcinomas, designated SCC-IV and SCC-VII; an ovarian adenocarcinoma, OCa-I; a hepatocarcinoma, HCa-I; and an adenosquamous carcinoma, ACa-SG) syngeneic to C3Hf/Kam mice using Western blot analysis. These tumors greatly differed in their radioresponse, assessed by TCD50 assay, and in their susceptibility to radiation-induced apoptosis. Likewise, the expression of EGFR greatly varied, by as much as 21-fold, and the magnitude of the EGFR expression positively correlated with increased tumor radioresistance. The levels of EGFR inversely correlated with radiation-induced apoptosis, suggesting that the lack of sensitivity to apoptosis induction was a major mechanism responsible for radioresistance of tumors with high EGFR. This correlation was highly significant only for wild-type p53 carcinomas. Radiation activated EGFR autophosphorylation and increased the activity of protein tyrosine kinase, but only in tumors with high EGFR expression. Thus, EGFR expression was a major determinant of tumor radioresponse in vivo. The pretreatment assessment of EGFR expression could predict radiotherapy outcome and may assist in selecting an effective treatment modality.

INTRODUCTION
The EGFR is a 170,000 transmembrane glycoprotein with an intracellular domain possessing intrinsic tyrosine kinase activity. On binding to a ligand, such as EGF or transforming growth factor α, EGFR is autophosphorylated and initiates transduction signals that regulate cell division, proliferation, and differentiation (1, 2). This receptor is frequently expressed at high levels in many types of cancer and is often associated with more aggressive tumors, poor prognosis, and resistance to treatment with cytotoxic agents, including ionizing radiation (2–6). In vitro experimental studies have yielded solid evidence linking EGFR with resistance to cytotoxic drugs (2, 7–10). Transfection of EGFR into human breast cancer cells, for example, was reported to increase cellular resistance to drugs (7). On the other hand, blockade of the EGFR-mediated signaling pathway with antibodies to EGFR enhanced the sensitivity of tumor cells to a number of chemotherapeutic agents (2, 8, 9). More recent studies have shown that anti-EGFR antibodies are effective in the treatment of human tumor xenografts, particularly when combined with chemotherapeutic drugs (10). The approach of combining anti-EGFR antibody with cytotoxic agents in the treatment of cancer patients is presently under testing (2, 11).

Studies on the role of EGFR and its ligands in tumor response to radiation are scarce and limited to in vitro experiments. EGF was reported to either enhance (12–14) or inhibit (15, 16) the cytotoxic effect of ionizing radiation. Kwok and Sutherland (12, 13) observed that EGF increased radiosensitivity of squamous cell carcinoma cell lines that had high-affinity surface EGFR. The radioenhancement was most significant in G1-phase cells and was associated mainly with a reduction of the shoulder region of the dose-survival curve. There was an inverse relationship between the number of high-affinity EGFRs and the degree of EGF radiosensitization (12). Another study showed that the magnitude of radioenhancement induced by EGF depended on the intrinsic radiosensitivity of tumor cells, with the effect being greater in more radiosensitive cells (14).

In contrast, a number of studies showed that EGF protected tumor cells against radiation killing (15, 16). Wollman et al. (15) observed that the addition of EGF to cultures of hormone-deprived MCF-7 carcinoma cells before irradiation increased their radioresistance, and this effect was abrogated by a specific antibody to EGFR. The resistance was associated with an accumulation of cells in the radioresistant S-phase of the cell division cycle as well as in an elevation of the intracellular glutathione content. Balaban et al. (16) showed that the presence of EGF during and after irradiation decreased the radiosensitivity of A431 carcinoma cells, whereas exposing cells to monoclonal antibodies against EGFR increased sensitivity by enhancing radiation-induced apoptosis. It was concluded that in A431 cells, radiation predominantly activates EGFR and thereby initiates downstream processes leading to radioresistance (16).

Our study was undertaken to assess the relationship between the extent of EGFR expression and radiocurability of murine tumors in vivo. Upon establishing a significant correlation between tumors with higher levels of EGFR and radioresistance, a series of investigations were initiated to evaluate the...
pathways by which EGFR overexpression may affect in vivo tumor response to ionizing radiation.

**MATERIALS AND METHODS**

**Mice and Tumors.** Inbred C3Hf/Kam mice, bred and maintained in our specific-pathogen-free mouse colony, were used in this study. They were 9–12 weeks of age when used for the experiments, and they were housed five/cage. Nine different murine carcinomas syngeneic to C3Hf/Kam mice were used: four mammary carcinomas (designated MCa-4, MCa-29, MCa-35, and MCa-K), two squamous cell carcinomas (SCC-IV and SCC-VII), an ovarian adenocarcinoma (OCa-I), a hepatocarcinoma (HCa-I), and an adenosquamous carcinoma (ACa-SG). Solitary tumors were generated by inoculating 5 $\times$ 10$^6$ viable tumor cells into thigh muscles of the right leg of mice. Tumor cell suspensions were prepared from tumor source animals by excision of the tumor, mechanical disruption, and enzymatic digestion of nonnecrotic tumor tissue (17).

**Tumor Radioresponse.** A TCD$_{50}$ assay was used to assess tumor response; the TCD$_{50}$ constitutes the dose of radiation yielding local tumor control in 50% of irradiated animals. The TCD$_{50}$s were reported earlier for seven of the nine tumors (18–20). Both in the earlier study and in this one, tumors of 8 mm in diameter were locally irradiated with a range of single doses of $\gamma$-irradiation, delivered from a dual-source $^{137}$Cs irradiator at a dose rate of 6.3 Gy/min. During irradiation, anesthetized mice were immobilized on a jig, and the tumor was centered in a 3-cm diameter radiation field. Mice were checked for the presence of tumor at the irradiated site at 9- to 12-day intervals for up to 120 days. The TCD$_{50}$s, computed by logit analysis (21), were: 42.9 (38.4–45.5) Gy for MCa-K; 52.6 (49.3–56.0) Gy for OCa-I; 58.3 (53.3–65.1) Gy for MCa-35; 62.3 (56.7–68.5) Gy for MCa-29; 64.7 (63.9–65.6) Gy for MCa-4; 65.4 (57.7–74.1) Gy for ACa-SG; 67.6 (64.4–71.1) Gy for SCC-IV; ~80 Gy for SCC-VII; and >81 Gy for HCa-I. In parentheses are 95% confidence limits.

**Histological Analysis of Apoptosis.** Tumors 8 mm in diameter were exposed to a single 25-Gy dose of $\gamma$-rays and 3 h later analyzed for the percentage of apoptotic cells. We reported previously (22, 23) that radiation-induced apoptosis in these tumors peaks at 3–6 h after tumor irradiation and returns to near background within 1–2 days. Briefly, five nongneecrotic fields on each H&E-stained histological section were randomly selected and examined micromorphometrically at $\times$400. One hundred nuclei in each field were scored as normal or apoptotic in appearance, and the results were expressed as a percentage based on counting 1500–2000 nuclei/group. The percentages of radiation-induced apoptosis were reported earlier (24) for all tumors in this study with the exception of ACa-SG. The mean percentage values and their SEs were: 0% for MCa-K and HCa-I; 0.2% for SCC-VII; 0.4% for SCC-IV; 9.1 $\pm$ 0.1% for ACa-SG; 14.0 $\pm$ 1.7% for MCa-35; 14.6 $\pm$ 1.4% for MCa-29; 22.9 $\pm$ 3.5% for OCa-I; and 24.3 $\pm$ 0.2% for MCa-4 tumor.

**Western Blotting.** EGFR expression was determined in nine different carcinomas by Western blot analysis. Samples consisting of 8–9-mm tumors and cut into tiny pieces, washed three times in ice-cold PBS (pH 7.4), and lysed in a buffer containing 2% SDS, 20% glycerol, 10 mg/ml PMSF, and leupeptin (Sigma Chemical Co., St. Louis, MO). The lysates were then denatured at 100°C for 5 min in the presence of 5% β-mercaptoethanol and loaded onto 7 or 12% polyacrylamide gels. Proteins were fractionated by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) in a transfer buffer consisting of 48 mM Tris base, 20% methanol, 0.04% SDS, and 30 mM glycine.

The membranes were incubated with polyclonal EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 3 h at room temperature after incubation with a blocking buffer consisting of PBS containing 0.1% Tween 20 (PBST) and 3% skim milk (Amersham International, Little Chalfont, United Kingdom). After incubation with biotinylated anti-rabbit IgG or anti-mouse IgG (Dako Corp., Carpenteria, CA) for 60 min, the membranes were incubated with peroxidase-conjugated streptavidin (Dako Corp.) for 30 min. The ECL Western blotting detection system (Amersham International) was applied for chemiluminescence detection. After each incubation period, the membranes were washed three times with PBST. Total cellular proteins applied to each lane were adjusted to equal concentrations with BCA protein assay reagent (Pierce, Rockford, Illinois). Detectable proteins were quantified by densitometry using a Du-70 spectrophotometer (Beckman Instruments, Fullerton, CA).

**Immunoprecipitation.** Tumor tissue was lysed with cold RIPA buffer [1.25% NP40, 1.25% sodium deoxycholate, 0.0125 m sodium phosphate (pH 7.2), and 2 mM EDTA] containing phosphatase inhibitors (0.2 mM sodium vanadate and 50 mM sodium fluoride) and proteinase inhibitor (aprotinin, leupeptin, and PMSF) using tissue cylinder. Three hundred mg of total proteins were immunoprecipitated with 5 ml of anti-rabbit polyclonal EGFR antibody (Santa Cruz Biotechnology), followed by protein A- and protein-G-Sepharose beads (Sigma). Normal rabbit IgG antibody was used as a negative control. The immunoabsorbed pellets were washed two times with cold RIPA buffer and one time with PBS (136 mM NaCl, 2.6 mM KCl, 8 mM Na$_2$HPO$_4$, and 1.4 mM KH$_2$PO$_4$, and then resuspended in 50 ml of 2× SDS [1× SDS = 250 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromphenol blue, and 2% β-mercaptoethanol] sample buffer. After being denatured at 100°C for 5 min, the samples were resolved on SDS-PAGE gels. To detect tyrosine phosphorylation of EGFR, anti-phosphotyrosine antibody (Santa Cruz Biotechnology) was used as primary antibody, and subsequent procedures were as described above.

**Protein Tyrosine Kinase Activity Assay.** Total cellular protein tyrosine kinase activity was measured using a commercial assay kit (Oncogene Research Product, Cambridge, MA) according to the manufacturer’s instructions. Tumor tissues were lysed in 2 ml of extraction buffer [20 mM Tris (pH 7.4), 50 mM NaCl, 1 mM EGTA, 1 mM EDTA, and 0.2 mM PMSF], and 1-mg samples of each was added to wells coated with protein tyrosine peptide substrate. The same volume of Abl tyrosine kinase was used as a positive control. The kinase reaction was carried out at room temperature for 30 min by adding 90 μl of kinase reaction buffer containing 0.1 mM ATP. After wells were washed by washing buffer containing 0.1% Tween 20 and 0.1% 2-chloroacetic acid, each well was incubated with horseradish peroxidase-conjugated PY-20 antibody for 30 min at room temperature and 100 ml of the chromogenic

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substrate for 6 min in the dark. The reaction was stopped by adding stop solution containing 2.5 N H₂SO₄, and the absorbency of each well was measured at 450 nm using a spectrophotometer.

RESULTS

Nine different carcinomas were examined for EGFR protein expression using Western blot analysis. The expression greatly differed among the tumors (Fig. 1), with the mean relative densitometric value ranging from 1 (MCa-K tumor) to 21 (SCC-VII tumor). However, EGFR expression within each carcinoma type showed little variability (Fig. 2). Three tumors of each tumor type grown to 8–9 mm in diameter were used for analysis. In general, we observed that tumor size between 8 and 12 mm in diameter did not influence the level of EGFR expression (data not shown).

We reported earlier (18–20) that these carcinomas exhibit wide variation in radiation response, i.e., the TCD₅₀ ranged from 42.9 (38.4–45.5) Gy for MCa-K to more than 81 Gy for HCa-I tumor. To examine whether there exists a relationship between in vivo tumor radioresponse and EGFR expression, the correlation between TCD₅₀ of these tumors and the tumors’ EGFR expression levels was determined. As shown in Fig. 2A, there was a significant \( r = 0.8, P < 0.01 \) inverse correlation between the magnitude of EGFR expression and tumor radiocurability. Seven of the nine tumors possessed wt p53, whereas two, MCa-K and MCa-35, had mutant p53. When the correlation between EGFR expression and radioresponse of only wt p53 tumors was determined, it remained significant \( r = 0.79, P = 0.04 \); Fig. 2B).

A number of mechanisms determine tumor response to irradiation, including sensitivity of tumor cells to apoptosis induction. Our own studies (22–24) showed that murine tumors were more responsive to radiation if their cells were sensitive to radiation-induced apoptosis. On the other hand, many growth factors, including EGF, promote survival of cells and can increase cell resistance to cytotoxic agents, including ionizing radiation (1, 2). Therefore, a likely possibility accounting for the inverse correlation between tumor radiocurability and EGFR expression is that cells of tumors with high EGFR expression levels have low sensitivity to apoptosis induction by radiation. Fig. 3 correlates the EGFR expression levels with the percentage of radiation-induced apoptosis. Apoptosis was determined 3 h after tumor irradiation with 25 Gy (24). With the exception of MCa-K, tumors with higher levels of EGFR had lower percentages of radiation-induced apoptosis. The correlation was borderline significant \( r = 0.66, P = 0.055 \) for all tumors (Fig. 3A) but was highly significant \( r = 0.93, P = 0.002 \) for tumors with

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**Fig. 1** EGFR protein expression (top panel) in nine murine carcinomas (Western blotting). Densitometric values range from 1 (MCa-K) to 21 (SCC-VII). Lower panel, actin expression.

**Fig. 2** Relationship between EGFR expression and radiocurability of murine carcinomas. A, both wt and mutant p53 (○) tumors; B, wt p53 tumors only. Bars, SE. In some symbols, SEs are too small to be seen.
wt p53 (Fig. 3B). Fig. 4 shows that for this latter group of tumors, there was also a significant correlation between the radiation-induced apoptosis and TCD_{50}. It should be noted that the MCa-K tumor was rather peculiar in that it was most sensitive to radiation, and yet its cells were resistant to radiation-induced apoptosis. This tumor is the only immunogenic tumor (25); therefore, it was excluded. In that case, the correlation between radiation-induced apoptosis and TCD_{50} and between radiation-induced apoptosis and EGFR expression became highly significant: \( P = 0.02 \) and \( P < 0.0001 \), respectively.

To initiate cellular transduction signals, EGFR must undergo autophosphorylation, and a number of in vitro studies show that ionizing radiation can induce EGFR autophosphorylation (26–28). We tested here whether tumor irradiation in vivo induces EGFR autophosphorylation and whether the induction depends on the basal level of EGFR expression. OCa-I, a low EGFR-expressing tumor, and SCC-VII, a high EGFR-expressing tumor, were exposed to 15 Gy of local tumor irradiation and 1, 2, 3 and 4 h later assessed for EGFR phosphorylation. Fig. 5 shows that radiation induced phosphorylation of EGFR in SCC-VII but not in OCa-I tumor. The EGFR phosphorylation occurred within 1 h after irradiation and persisted for the observation period of 4 h. Thus, radiation induced phosphorylation of EGFR only when the basal level of the receptor was high. Radiation, however, did not influence the basal level of EGFR expression (Fig. 5).

Because EGFR-mediated signal transduction depends on the activity of PTK, we determined total cellular PTK activity in tumors with low (MCa-4 and MCa-29 tumors) or high (SCC-VII and HCa-I tumors) EGFR levels before and up to 24 h after 15 Gy of irradiation. Fig. 6 shows that the basal PTK activity was about twice as high in tumors with high EGFR levels as in tumors with low EGFR levels. Irradiation induced a significant transient increase in PTK activity in tumors with high EGFR levels. The increase was about two times at 1 h after irradiation. At 24 h after irradiation, the PTK activity in the SCC-VII tumor returned to the background level, whereas in the HCa-I tumor, it still remained elevated. In contrast to the effect on PTK activity in tumors with high EGFR levels, in tumors with low EGFR levels irradiation either had no effect on PTK activity (MCa-4) or reduced PTK activity (MCa-29).

**DISCUSSION**

The outcome of tumor treatment with radiotherapy or other cytotoxic agents is determined by the actions and interactions of various intrinsic genetic factors such as p53 and bcl-2 oncogenes (29–31) and extrinsic epigenetic factors including growth factors, cytokines, and tumor oxygenation status (2, 32, 33). As mentioned earlier, a number of studies found that EGFR-overexpressing tumors were more virulent, conferred a poorer prognosis, or were more resistant to cytotoxic compounds (2–6, 34, 35). Evidence is also emerging suggesting that EGFR and its ligands affect the susceptibility of cells to apoptosis. Pierce et al.
demonstrated that transfection of EGFR into hematopoietic cells conferred resistance to apoptosis induction. In contrast, treatment of A431 cancer cells with anti-EGFR monoclonal antibodies sensitized them to radiation-induced apoptosis (16).

To our knowledge, we are the first to show that a highly significant inverse relationship exists between the magnitude of EGFR expression and the in vivo radiocurability of nonimmunogenic mouse carcinomas (P < 0.01). Our study also revealed a highly significant inverse correlation between the magnitude of EGFR expression and the extent of radiation-induced apoptosis (P < 0.0001). Recently, an association between EGFR expression and in vitro resistance to 2 Gy of single-dose radiation of primary cultures derived from 14 head and neck carcinoma patients was reported (37). Tumor resistance was measured by the extent of radiation-induced cell growth inhibition. That EGFR and its ligands may actively regulate cell response to radiation is shown by studies that cells exposed to EGF are protected from radiation damage (15, 16), whereas those pretreated with monoclonal antibodies against EGFR are radiosensitized to damage (15, 16).

Many studies have shown that p53 mutation is frequently associated with resistance to cytotoxic treatments (29, 31, 38), and some evidence suggests an association between p53 mutation and EGFR overexpression (39–42). We found, however, that the inverse relationships between EGFR overexpression and radiocurability or propensity for induced apoptosis remained highly significant for tumors with wt p53. This implies that EGFR may influence apoptotic response independently of p53 function and involve processes essential for cell survival, such as cell cycle regulators including cyclin D, p27, and p21 (43). Overall, our studies suggest that the lack of radiation to induce apoptosis in tumors with high expression of EGFR may be one of the mechanisms of tumor radioresistance.

EGFR must undergo autophosphorylation to generate transduction signals leading to coregulation of cell division, proliferation, and differentiation. Several in vitro studies demonstrated that ionizing radiation mimics the action of ligand-receptor binding, which triggers downstream signaling (16, 26–28, 44). Balaban et al. (16), for example, showed that radiation affects multiple signaling pathways, but induction of radioresistance is predominantly associated with the activation of EGFR. Schmidt-Ullrich et al. (26, 27) reported that irradiation of human mammary and squamous carcinoma cell lines induces EGFR autophosphorylation, followed by activation of transduction pathways, including Raf-1 and mitogen-activated protein kinase. Blockade of EGFR autophosphorylation by the specific inhibitor tyrphostin AG1478 abolished the transduction signal (27). The investigators postulated that activation of EGFR-mediated signals stimulates cell proliferation, which may underlie acceleration in tumor clonogen repopulation during the course of fractionated radiotherapy.

The data of our study suggest that radiation-induced EGFR autophosphorylation may depend on the basal level of EGFR expression because autophosphorylation occurred in a high EGFR-expressing tumor but not in a low EGFR-expressing tumor. Similarly, a radiation-induced transient increase in PTK activity occurred in tumors with a high basal PTK activity, whereas no change or even a transient reduction in PTK activity took place in tumors with low basal PTK level. These observations suggest that a certain basal level of EGFR must be present to respond to radiation to activate downstream processes leading to cell protection. The protection in our study was manifested in the lower susceptibility to radiation-induced apoptosis. There-
fore, inefficiency of radiation to induce apoptosis in tumors with high levels of EGFR may be one of the mechanisms of tumor radioresistance. Another mechanism would involve influence on tumor cell regeneration. Whether high levels of EGFR would result in sooner onset and more rapid subsequent regeneration of tumor clonogens that survived tumor irradiation is not known, but answers to these questions require fractionation irradiation type experiments, which are presently under way in our laboratory. It should be noted that in our studies, a high-dose radiation (15 Gy) was used to demonstrate induction of EGFR phosphorylation. This dose was used to be close to radiation doses used for apoptosis induction and tumor radiosensitivity studies. Radiation-induced EGFR autophosphorylation was reported previously to occur after a clinical dose range of irradiation (26).

Our findings may have important clinical implications. The pretreatment assessment of EGFR expression could predict radiotherapy outcome and could assist in selecting an effective radiotherapeutic approach to radioresistant tumors. Also, radiotherapy could be combined with agents that block EGFR and/or its downstream signaling processes. Relevant to this is an ongoing clinical trial that combines anti-EGFR monoclonal antibodies with radiotherapy in the treatment of head and neck cancer (11). The preliminary results of this trial are highly encouraging, showing that those anti-EGFR antibodies greatly increase the percentage of local tumors controlled by radiotherapy. Also, we observed recently that the same antibody (C225) used in the above trial greatly enhanced radioresponse of A431 human tumor xenografts in nude mice. Thus, the present study and other recently published studies show that EGFR expression may be a biologically important determinant of tumor response to cytotoxic treatments.

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