Epidermal Growth Factor Receptor Blockade with C225 Modulates Proliferation, Apoptosis, and Radiosensitivity in Squamous Cell Carcinomas of the Head and Neck

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ABSTRACT
We examined effects of the anti-epidermal growth factor receptor monoclonal antibody C225 on proliferation, cell cycle phase distribution, apoptosis, and radiosensitivity in squamous cell carcinoma (SCC) cell lines derived from head and neck cancer patients. Exposure to C225 in culture inhibits SCC proliferation in a time-dependent manner, and the degree of growth inhibition, compared to controls, ranges from 20 to 75%. Flow cytometry analysis demonstrates that C225 treatment induces accumulation of cells in G1, which is accompanied by a 2–3-fold decrease in the percentage of cells in S phase. C225 exposure also induces apoptosis in SCC populations, as demonstrated by flow cytometry analysis using dual stainings of merocyanine 540 and Hoechst 33342. Western blot analysis indicates that C225 exposure induces accumulation of hypophosphorylated retinoblastoma protein and increases expression of p27KIP1. An increase in Bax expression and concurrent decrease in Bcl-2 expression are observed when SCC cells are exposed to C225. Examination of C225 effects on radiation response in SCCs demonstrates enhancement in radiosensitivity and amplification of radiation-induced apoptosis. These effects are observed in both single-dose and fractionated radiation experiments. C225 represents a promising growth-inhibitory agent that can influence cellular proliferation, apoptosis, and radiosensitivity in SCCs of the head and neck.

INTRODUCTION
SCCs of the H&N are among the most rapidly proliferating human solid tumors, with median tumor potential doubling times of 3–5 days measured in vivo. Radiation therapy plays a primary role in treatment for patients with SCC of the H&N. However, the rapid proliferation of surviving tumor clonogens during a conventional 7–8-week course of H&N radiotherapy serves to compromise ultimate tumor control (1). Intensified radiotherapy or chemotherapy regimens delivered over a reduced treatment time represent one approach to counteract rapid tumor repopulation (2). Modest clinical gains have been demonstrated with such treatment schedules, although they are generally accompanied by a prominent increase in acute toxicity (3). An alternative biological approach, which uses antiproliferative agents such as the anti-EGFR mAb C225 to alter tumor proliferation and/or radiosensitivity, therefore, offers promise for improving tumor control (4).

The EGFR system represents a promising therapeutic target because it is commonly overexpressed in human epithelial tumors (5). Elevated levels of EGFR and its ligand, TGF-α, have been identified in the majority of primary tumors from patients with H&N SCCs (6). This EGFR/TGF-α overexpression has been shown to correlate with aggressive malignant progression and poor clinical outcome (7, 8). In addition, a positive relationship between EGFR/TGF-α expression and proliferation of surviving tumor cells after radiation therapy has been documented (9). Studies have reported that cell survival and repopulation may be regulated by the activation and expression of EGFR/TGF-α that is induced following radiation (10, 11). In addition, the activation of downstream effectors of the EGFR signaling pathway, i.e., ras and raf oncogenes, have been shown to increase cellular resistance to ionizing radiation (12). These findings suggest that EGFR blockade may be useful in reducing tumor cell repopulation by inhibition of tumor cell growth and/or modulation of cellular radiosensitivity.

C225 is a mouse-human chimeric anti-EGFR mAb. Blockade of ligand binding to EGFR by C225 inhibits activation of the receptor tyrosine kinase (13). In addition, C225 has been shown to inhibit the proliferation of a variety of cultured malignant human cell lines that overexpress EGFR, including cells from vulva, breast, colon, lung, kidney, and prostate (14, 15). Inhibition of cell growth may result from the induction of cell cycle arrest and/or induction of apoptosis (16, 17). In addition, treatment with C225 has been shown to augment the antitumor activity of several chemotherapeutic drugs in mouse xenograft models (18). The objective of this study was to examine the potential therapeutic utility of C225 in conjunction with radiation to counteract tumor cell repopulation and to modulate cellular radiosensitivity and apoptosis in SCCs of the H&N.

MATERIALS AND METHODS
Chemicals and C225. Cell culture media were obtained from Life Technologies, Inc. (Gaithersburg, MD). PI and Hoe342 were obtained from Molecular Probes (Eugene, OR). Primary antibodies against Rb, p27KIP1, Bcl-2, and Bax were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-α-tubulin antibody was obtained from Oncogene Research Products (Cambridge, MA). ECL chemiluminescence detection system was purchased from Amersham (Arlington Heights, IL). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). C225 was generously provided by ImClone Systems Inc. (New York, NY).

Cell Lines and Cell Culture. Human SCC cell lines were established from biopsies of H&N cancer patients. The SCC-13Y cell line was derived from the facial epidermis and was provided by Dr. B. Lynn Allen-Hoffman (University of Wisconsin, Madison, WI). The SCC-1 (floor of mouth), SCC-11B (hypopharynx), and SCC-38 (tonsil) cell lines were obtained from Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI). SCC cells were cultured routinely in DMEM supplemented with 10% FCS serum (HyClone, Logan, UT), 1 μg/ml hydrocortisone, 1% penicillin, and 1% streptomycin.

Growth Inhibition. The antiproliferative effect of C225 on the in vitro growth profile of each SCC cell line was examined following replicate plating of known numbers of single cells from each cell line into 100-mm culture dishes. After 24 h, 30 nm C225 was added to the medium; cells were then trypsinized and counted with a hemacytometer at indicated time intervals.

Flow Cytometric Analysis. Control or C225-treated cells were harvested by trypsinization, washed with PBS, and then fixed in 95% ethanol and stored at 4°C for up to 7 days prior to DNA analysis. After the removal of ethanol by centrifugation, cells were then incubated with phosphate-citric acid buffer [0.2 M NaHPO4 (pH 7.8)–4 mM citric acid] at room temperature for 45 min. After centrifugation, cells were then stained with a solution containing 33 μg/ml PI, 0.13 mg/ml RNase A, 10 mM EDTA, and 0.5% Triton X-100 at 4°C for 24 h.

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The abbreviations used are: SCC, squamous cell carcinoma; H&N, head and neck; EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; TGF-α, transforming growth factor-α; PI, propidium iodide; Hoe342, Hoechst 33342; FC, Fetal Clone-II; MCF540, merocyanine 540; CDK, cyclin-dependent kinase; EGF, epidermal growth factor; DNA PK, DNA-dependent protein kinase.

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Stained nuclei were analyzed for DNA-PI fluorescence using a Becton Dickinson FACScan flow cytometer. Resulting DNA distributions were analyzed by ModFit (Verity Software House Inc., Topsham, ME) for the proportion of cells in apoptosis and in the G0/G1, S, and G2-M phases of the cell cycle.

**Apoptosis Analysis.** Apoptosis was evaluated following dual staining of SCC cells with Ho342 and MC540, as described previously (19). Ho342 is a DNA-specific dye that allows for the analysis of cell cycle position, and MC540 detects membrane phospholipid changes that occur in apoptotic cells. Briefly, cells were harvested with 5 mM EDTA at 37°C. After centrifugation, cell pellets were resuspended in 900 μl of PBS, followed by addition of 100 μl of 50 μg/ml Ho342. Thereafter, cells were incubated for 30 min in the dark, pelleted, and resuspended in 100 μl of PBS. Four μl of MC540 (1 mg/ml) were added, and cells were incubated for 20 min in the dark. Cells were pelleted, resuspended in 1 ml of PBS, and analyzed immediately by flow cytometry.

**Immunoblotting Analysis.** After treatment, cells were lysed with Tween 20 lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Tween 20, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 25 μg/ml aprotinin] and sonicated. Equal amounts of protein were analyzed by SDS-PAGE. Thereafter, proteins were transferred to nitrocellulose membranes and analyzed by specific primary antibodies against Rb, p27^KIP1, Bcl-2, or Bax. Proteins were detected via incubation with horseradish peroxidase-conjugated secondary antibodies and the ECL chemiluminescence detection system.

### RESULTS

**C225 Inhibits SCC Proliferation.** Growth curve profiles were evaluated in each SCC cell line following the addition of 30 nM C225 directly to the culture medium. These growth inhibition profiles over an 8-day exposure period are depicted in Fig. 1. Exposure to C225 inhibited cellular proliferation of SCC-1, SCC-11B, SCC-38, and SCC-13Y cells in a time-dependent manner. Growth inhibition of SCC cell lines by C225, compared with that of untreated controls, ranged from 20 to 75%.

**C225 Induces G0/G1 Arrest.** The capacity of C225 to inhibit cell cycle progression was evaluated via flow cytometry. A representative example depicting the effect of C225 treatment on cell cycle phase distribution in the SCC-13Y cell line is summarized in Fig. 2. Treatment with 30 nM C225 for 2 days resulted in the accumulation of cells in the G0/G1 phase (64.6%) compared with that in controls (54.4%). A concurrent decrease in the percentage of cells in S phase was observed in C225-treated cells (8.0%) relative to that of the controls (23.1%). The G0/G1 accumulation and S-phase depletion were even more pronounced in cells treated with C225 for 4 days (70.5% and 4.5%, respectively). No significant changes in the percentage of cells within G2-M phase were observed in these experiments.

To further characterize cell cycle events that might account for the observed G0/G1 arrest, we examined the effect of C225 on the expression of several key regulators of the G1-S phase transition. As shown in Fig. 4, immunoblotting analysis showed that treatment with 30 nM C225 increased the level of p27^KIP1, which is an inhibitor of G1 CDKs. In addition, treatment with C225 induced accumulation of the hypophosphorylated form of the Rb protein. The Rb protein is one of the critical substrates for G1 CDKs. Phosphorylation of Rb (hyperphosphorylated Rb) and the subsequent release of transcription factor E2F are required for G1-S transition. Therefore, our results suggest that treatment of SCC cells with C225 may induce G0 arrest by increasing levels or activity of the CDK inhibitor, which results in subsequent accumulation of hypophosphorylated Rb.
C225 Induces Apoptosis. To determine whether C225 influenced SCC apoptotic response, we exposed SCC-13Y cells to 30 nM C225 for various intervals and analyzed for apoptosis by flow cytometry using Ho342 and MC540 staining. As described previously, Ho342 is a DNA-specific dye that allows for analysis of cell cycle position and MC540 detects membrane phospholipid changes which occur in apoptotic cells. Dual staining of Ho342 and MC540 enables the identification of five distinct subpopulations (R2–R6) that are MC540 negative/dull: R3, cells with greater than 2 N DNA that are MC540 negative/dull; R4, cells with 2 N DNA that are MC540 bright; R5, cells with greater than 2 N DNA that are MC540 bright; R6, cells with reduced Ho342 staining (fragmented DNA) and MC540 bright. B, 6–7-fold increase in the fragmented DNA subgroup R6 (corresponding to latter phase of apoptosis) with longer exposure duration to C225.

C225 Induces Apoptosis. To determine whether C225 influenced SCC apoptotic response, we exposed SCC-13Y cells to 30 nM C225 for various intervals and analyzed for apoptosis by flow cytometry using Ho342 and MC540 staining. As described previously, Ho342 is a DNA-specific dye that allows for analysis of cell cycle position and MC540 detects membrane phospholipid changes which occur in apoptotic cells. Dual staining of Ho342 and MC540 enables the identification of five distinct subpopulations, R2–R6. Subpopulations R2 and R3 represent viable cells, R4 and R5 represent early apoptotic cells, and R6 represents cells undergoing the latter stages of apoptosis by fragmenting their DNA. As shown in Fig. 3A, exposure of SCC-13Y to 30 nM C225 gradually increased cell numbers within the R4–R6 subpopulations with a concurrent decrease in the viable cell populations (R2 and R3). More specifically, a significant accumulation of cells (6–7-fold) in the R6 subpopulation was observed when cells were exposed to C225 for 72 h (Fig. 3B). In contrast, no significant changes in the distribution of cells among the five subpopulations were observed in control groups over a 72-h evaluation. These results suggest that treatment of SCC-13Y cells with C225 not only induces G0/G1 cell cycle arrest but also induces apoptosis.

To further investigate the induction of apoptosis by C225, we conducted immunoblotting analysis to examine the expression of Bcl-2 and Bax, which are the critical regulators of apoptosis. In general, it is known that Bcl-2 protects cells from apoptosis, whereas Bax promotes apoptosis. The propensity of a cell to undergo apoptosis is dependent in part on the Bax:Bcl-2 ratio within the cell (20). As shown in Fig. 4, treatment of SCC-13Y cells with C225 reduced Bcl-2 expression and increased Bax expression. Consistent with the previous analysis of apoptosis using Ho342/MC540 staining, these results support the finding that exposure of SCC-13Y to C225 increases the Bax:Bcl-2 ratio and promotes the progression of cells to apoptosis.
C225 MODULATION OF PROLIFERATION AND RADIATION RESPONSE

C225 Enhances Radiosensitivity. To examine the potential usefulness of combining C225 with radiation therapy for rapidly dividing human SCCs of the H&N, we conducted experiments to determine the effects of C225 on single-dose and fractionated radiation on the survival of SCC-13Y cells. Fig. 5 depicts radiation-survival curves for cells exposed to C225 either before or after exposure to radiation. Exposure of SCC-13Y cells to C225 alone (30 nM) induced a reduction in plating efficiency of ~15–20%. On a single-dose radiation schedule (Fig. 5, left), both preirradiation and postirradiation treatments with C225 increased the radiation cell kill. Exposure to C225 following radiation appeared to be slightly more effective in reducing cell survival compared with preirradiation treatment by C225. Similarly, the effect of C225 on clonogenic survival was investigated using a fractionated radiation treatment schedule (Fig. 5, right). In both the preirradiation and postirradiation exposure settings, treatment with C225 reduced cell survival significantly (P = 0.001, two-way ANOVA).

C225 Enhances Radiation-induced Apoptosis. To further examine the capacity of C225 to influence radiation-induced apoptosis, we harvested control and irradiated SCC-13Y cells and replated them in DMEM with 10% FC serum. After 3 days of incubation, cells were analyzed for sub-G _, apoptotic population by flow cytometric analysis, as described previously. Fig. 6A demonstrates these results following single-dose radiation exposure. Radiation alone (6 Gy) produced a 2-fold induction of apoptosis, whereas exposure to 30 nM C225 in combination with 6 Gy of radiation induced apoptosis 5–6-fold, compared to untreated controls. Treatment with 30 nM C225 alone induced apoptosis by ~2-fold. Similar results were found using the fractionated radiation treatment schedules (Fig. 6B).

DISCUSSION

The proliferation of tumor cells during radiation treatment has been identified as a factor that adversely impacts tumor response and ultimate local control (1–3). One approach to reduce the impact of tumor cell repopulation during treatment involves the delivery of biological agents that slow or inhibit tumor cell proliferation. Here, we investigated the capacity of the anti-EGFR mAb C225 to inhibit proliferative growth and also examined the capacity of C225 to modulate radiosensitivity in SCCs of the H&N.

Our results demonstrate that C225 inhibits growth across a variety of SCC cell lines (all cell lines tested to date). The extent of growth inhibition ranges from 20 to 75% (Fig. 1). Growth inhibition of SCCs by C225 is accompanied by an accumulation of cells in G _ and by the induction of apoptosis (Figs. 2 and 3). The G _ arrest correlates with an increase in the level of the cell cycle inhibitor p27KIP1, which is known to inactivate G _ CDKs (Fig. 4). We also observed that exposure of SCCs to C225 stimulates production of hypophosphorylated Rb, an increase in the expression of Bax protein, and a decrease in the expression of Bcl-2 (Fig. 4). These results are consistent with previous reports using a variety of other human tumor cell lines (16, 17, 21).

Many components of the biological response to ionizing radiation in mammalian cells are mediated through signal transduction, cell cycle regulation, and DNA repair pathways (22). Growth factors play an important role in modulating these cellular responses to radiation. Although the in vitro effects of EGF on the radiosensitivity of tumor cells have been extensively studied, inconsistent results have been demonstrated. For example, Wollman et al. (23) showed that preirradiation exposure to EGF enhanced radioresistance in MCF-7 cells. In contrast, Kwok and Sutherland (24) reported that the presence of EGF before, during, or after irradiation enhanced radiosensitivity in CaSki cells. Additional studies demonstrated that, whereas some squamous cancer cell lines are sensitized by EGF (e.g., A431, SCC-6, and HN5),

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**Fig. 4.** C225 exposure modulates expression of proteins involved in cell cycle regulation and apoptosis. Exponentially growing cells were treated with C225 (30 nM) for various time intervals and harvested. Following cell lysis, the lysates were processed for immunoblotting using antibodies directed against Rb, p27KIP1, Bcl-2, and Bax as described in “Materials and Methods.” Arrows, hyper- and hypophosphorylated forms of Rb (hyperRb and hypoRb, respectively). α-Tubulin serves as a loading control.

**Fig. 5.** C225 enhances radiosensitivity. The influence of 30 nM C225 on intrinsic radiosensitivity was examined by clonogenic survival in SCC-13Y cells following exposure to single dose (left) or fractionated radiation (right) as described in “Materials and Methods.” For single-dose experiments, cells were exposed to C225 for 3 days before irradiation (pretreatment) or exposed to C225 during and after irradiation (posttreatment). For fractionated experiments, cells were exposed to C225 for 3 days followed by daily irradiation at 0, 1, 2, or 3 Gy for 3 consecutive days (pretreatment) or were irradiated in the presence of C225 for 3 days (posttreatment). Control samples are cells exposed to radiation without C225 treatment. Data points, means of duplicates with five cultures per replicate per point; bars, SD.
others are unaffected by the presence of EGF following radiation (e.g., SiHa and SCC-1; Refs. 25 and 26). One hypothesis regarding the aforementioned differential responses to EGF across various cancer cell lines is that cells that are growth stimulated by EGF are radio-protected, whereas those that are growth inhibited by EGF are radiosensitized (23). However, the underlying mechanisms remain to be determined.

Our results indicate that exposure to C225, either before or following radiation, enhanced the radiosensitivity of SCC-13Y cells (Fig. 5). This finding is reproducibly observed with both single-dose and fractionated radiation treatment schemes. C225 exposure also enhanced radiosensitivity in SCC-38 cells under the same experimental conditions (data not shown). It is possible that the enhancement of radiosensitivity by C225 is mediated by mechanisms involving DNA repair inhibition. Such speculation derives from recent findings by Bandyopadhyay et al. (27), who demonstrated that C225 but not EGF triggers a specific physical interaction between internalized EGFR and DNA-PK in the cytosol in a variety of cell types. Significant reduction in the level and activity of DNA-PK in the nucleus with concurrent increase in DNA-PK levels in the cytosol were observed.

Fig. 6. C225 enhances radiation-induced apoptosis. Control or C225 treated SCC-13Y cells were either irradiated with single doses of 6 Gy (A) or irradiated daily at 2 Gy for 3 consecutive days (B). Following radiation, cells were replated, incubated for 3 days and processed for flow cytometric analysis as described in “Materials and Methods.” DNA histograms of cells either untreated (Control), treated with C225 prior to irradiation (Pretreatment), or treated with C225 after irradiation (Post-treatment) are shown. Percentages of apoptosis were determined on the basis of sub-G0 DNA content (M1) of the histogram.

DNA Content

DNA Content

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Because DNA-PK is believed to play a major role in repairing DNA double-strand breaks, these findings suggest that C225 may impair DNA repair by reducing the nuclear level of DNA-PK.

The capacity of C225 to modulate tumor cell proliferation and cell cycle phase distribution may also play a central role regarding the observed enhancement of radiosensitivity. Prior studies have suggested that modulation of radiosensitivity by EGF is cell cycle dependent, with cells in G2 phase being more radiosensitive and cells in the S phase being more radiosensitive (28, 29). These results suggest that C225 may enhance radiosensitivity by inducing accumulation of cells in a more radiosensitive cell cycle phase (G2) and reducing the size of the radioreistant S-phase fraction. Another underlying mechanism that may contribute to the influence of C225 on radiosensitivity involves the availability and activity of free radical scavengers such as glutathione. Prior work in MCF-7 cells has identified that pretreatment exposure to C225 enhanced radiosensitivity, which was accompanied by a decrease in glutathione levels and the fraction of cells within S phase (23).

The success of radiation therapy in effectively local tumor control depends on several factors, including overall clonogenic burden, intrinsic radiosensitivity, oxygenation status, repopulation capacity, and repair. Apoptosis may also affect the ultimate tumor response to radiation by influencing the overall clonogenic burden through promotion of cell loss during treatment (30, 31). In tumor cells with apoptotic propensity, death may be triggered by lower radiation doses that than required to induce reproductive cell death. Our results indicate that treatment with C225 not only enhances reproductive cell death following radiation but also increases the fraction of tumor cells succumbing to radiation-induced apoptosis (Fig. 6).

In conclusion, findings from the present study indicate that C225 is an effective antiproliferative agent for SCCs of the H&N. Growth inhibition of SCC cells is mediated through the induction of G1 cell cycle arrest and the induction of apoptosis. In addition, we have confirmed that exposure of human SCCs to C225 enhances the effects of radiation therapy. Specifically, exposure to C225 enhances reproductive cell death and radiation-induced apoptosis in SCCs using both single-dose and fractionated radiation treatment schedules. Several biological and clinical characteristics of human SCCs of the H&N appear to lend themselves particularly well to the investigation of strategies that involve EGFR blockade plus radiation: (a) high EGFR expression levels, (b) rapid proliferative capacity, (c) radiation as a dominant treatment modality, and (d) locoregional recurrence as the dominant failure pattern. These characteristics in combination with the current findings suggest C225 as a promising agent for further investigation in the treatment of rapidly proliferating SCCs of the H&N. The potential therapeutic benefits of combining C225 with radiation therapy for patients with advanced SCCs of the H&N is scheduled to commence Phase III clinical trial evaluation in 1999.

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