Modulation of Radiation Response after Epidermal Growth Factor Receptor Blockade in Squamous Cell Carcinomas: Inhibition of Damage Repair, Cell Cycle Kinetics, and Tumor Angiogenesis

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Abstract
We have recently demonstrated that molecular blockade of the epidermal growth factor receptor with the anti-epidermal growth factor receptor (EGFR) monoclonal antibody C225 enhances the in vitro radiosensitivity of human squamous cell carcinomas (SCCs) derived from the head and neck. In the present study, we further investigated the capacity of C225 to modulate the in vitro and in vivo radiation response of human SCC tumor cells and xenografts, and we examined several potential mechanisms that may contribute to the enhanced radiation response induced by C225. Tumor xenograft studies demonstrated complete regression of both newly established (20 mm³) and well-established (100 mm³) SCC tumors over a 55–100 day follow-up period in athymic mice treated with the combination of C225 (i.p. injection) and radiation. Cell cycle analysis via flow cytometry confirmed that combined treatment with C225 and radiation induced an accumulation of cells in the more radiosensitive cell cycle phases (G1, G2-M) with concurrent reduction in the proportion of cells in the more radioresistant S phase. Results from sublethal damage repair and potentially lethal damage repair analyses in cultured SCC cells demonstrated a strong inhibitory effect of C225 on post-irradiation damage repair. Further, exposure of SCC cells to C225 induced a redistribution of DNA-dependent protein kinase from the nucleus to the cytosol, suggesting one potential mechanism whereby C225 may influence the cellular response to radiation. Immunohistochemical analysis of SCC tumor xenografts after systemic administration of C225 demonstrated inhibition of the in vivo expression of tumor angiogenesis markers, including vascular endothelial growth factor and Factor VIII. Taken together, the collective data suggest that the profound in vivo antitumor activity identified in the xenograft setting when C225 is combined with radiation derives from more than simply the antiproliferative and cell cycle effects of EGFR system inhibition. In addition to antiproliferative growth inhibition, EGFR blockade with C225 appears to influence the capacity of human SCCs to effect DNA repair after exposure to radiation, and to express classic markers of tumor angiogenesis.

Introduction
Locoregional disease recurrence remains the dominant form of treatment failure for patients with advanced SCC of the H&N. For patients treated with primary radiation therapy, methods to enhance locoregional disease control have commonly included trials with altered fractionation and trials in combination with cytotoxic chemotherapy (1–3). More recently, advances in our understanding and application of molecular biology to cancer therapy provide new opportunities to modulate tumor growth characteristics during treatment. One such approach in the treatment of SCC of the H&N involves modulation of radiation response by EGFR blockade using the anti-EGFR mAb C225 (4, 5). Recent studies have identified C225 as a potent antiproliferative agent in SCC of the H&N, capable of inhibiting tumor cell growth kinetics. In addition, preclinical studies have demonstrated the capacity of C225 to enhance in vitro radiosensitivity and to promote radiation-induced apoptosis (6).

Although C225 augmentation of antitumor activity for several chemotherapeutic agents in mouse xenograft models has been demonstrated (7–9), in vivo characterization of C225/radiation interactions have not been well established. Several preliminary findings regarding the capacity of C225 to inhibit cellular proliferation (10), to inhibit DNA damage repair (11), and to inhibit tumor angiogenesis (12) suggest mechanisms whereby EGFR blockade might enhance antitumor responses. The experimental studies presented herein were conducted to examine the in vivo response of SCC xenografts in athymic mice to dual treatment with radiation and C225, and to explore various mechanisms of C225-mediated enhancement of radiosensitivity.

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The abbreviations used are: SCC, squamous cell carcinoma; bFGF, basic fibroblast growth factor; DNA-PK, DNA-dependent protein kinase; EGFR, epidermal growth factor receptor; FVIII, factor VIII-related antigen; H&N, head and neck; mAb, monoclonal antibody; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; PLDR, potentially lethal damage repair; SLDR, sublethal damage repair; VEGF, vascular endothelial growth factor; PMSF, phenylmethylsulfonyl fluoride.
Materials and Methods

Chemicals and C225. Cell culture media were obtained from Life Technologies, Inc. (Gaithersburg, MD). PI was obtained from Molecular Probes (Eugene, OR). Primary antibody against VEGF, DNA-PK, and PCNA were obtained from Neo Markers (Fremont, CA). Antibody against FVIII (von Willebrand factor) was obtained from DAKO (Glostrup, Denmark). Anti-α-tubulin antibody was obtained from Oncogene Research Products (Cambridge, MA). The enhanced chemiluminescence detection system was purchased from Amersham (Arlington Heights, IL). All other chemicals were purchased from Sigma (St. Louis, MO). C225 was generously provided by ImClone Systems incorporated (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). The SCC-1 cell line (floor of mouth) and SCC-6 cell line (tongue) were provided by Dr. Thomas E. Carey (University of Michigan). The relative expression of EGFR on the cell surface of our SCC cell lines was evaluated via immunofluorescent staining with C225 and subsequent flow cytometry analysis. These SCC cell lines expressed EGFR at essentially comparable levels to those expressed in A431 cells, which are well known to overexpress the EGFR with several million receptors per cell. SCC cells were cultured routinely in DMEM supplemented with 10% Fetal Clone-II serum (Hyclone, Logan, UT), 1 μg/ml hydrocortisone, 1% penicillin, and streptomycin.

Assay of Tumor Growth in Athymic Nude Mice. Athymic Sprague Dawley nude mice (3–4-week-old females) were obtained from Harlan Bioproducts for Science (Indianapolis, IN) and maintained in a laminar air-flow cabinet under aseptic conditions. The care and treatment of experimental animals were in accordance with institutional guidelines. Human SCC cells (1–10⁶) were injected s.c. into the right (SCC-6) and left (SCC-1) flank area of the mice at day 0. Tumor volume was determined by direct measurement with calipers and calculated by the formula: \( \pi/6 \times (\text{large diameter}) \times (\text{small diameter})^2 \). Animal experiments included four treatment groups: control, radiation alone, C225 alone, and radiation in combination with C225. Control animals received injections of PBS. Radiation treatment was delivered via a precision electron beam from a Varian linear accelerator using custom-designed mouse jigs. These jigs immobilized the animals and specifically exposed the dorsal flank (harboring tumor xenografts) for irradiation without exposing non-tumor-bearing normal tissues. C225 was administered by i.p. injection at the specified doses and intervals.

Immunohistochemical Determination of PCNA, VEGF, and FVIII. The expression of proliferative and angiogenic factors were detected in histological sections of SCC xenografts.
Briefly, excised tumor specimens were fixed in 10% neutral-buffered formalin. After embedding in paraffin, 5-μm sections were cut, and tissue sections were mounted. Sections were dried, deparaffinized, and rehydrated. After quenching endogenous peroxidase activity and blocking nonspecific binding sites, slides were incubated at 4°C overnight with 1:100 dilution of primary antibody directed against PCNA, VEGF, or FVIII followed by a 30-min incubation of biotinylated goat antimouse secondary antibody. Slides were then incubated with streptavidin peroxidase and visualized using the DAB chromogen (Lab Vision Corp., Fremont, CA).

**Cell Cycle Analysis.** Cell cycle phase distribution after radiation and/or C225 treatment was analyzed by flow cytometry using PI staining. Briefly, control or treated cells were harvested by trypsinization, washed with PBS, then fixed in 95% ethanol and stored at 4°C for up to 7 days before DNA analysis. After the removal of ethanol by centrifugation, cells were incubated with phosphate-citric acid buffer [0.2 M Na₂HPO₄ and 4 mM citric acid (pH 7.8)] at room temperature for 45 min. 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. 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 proportions of cells in G₀-G₁, S phase, and G₂-M phases of the cell cycle.

**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 PMSF, and 10 μg/ml leupeptin and 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 DNA-PK. Proteins were detected via incubation with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence detection system.

**Subcellular Fractionation.** To examine the effect of C225 on the subcellular distribution of DNA-PK after radiation, control or C225-treated SCC cells were collected and separated into cytoplasmic and nuclear extracts. Briefly, after centrifugation, cells were resuspended in 150 μl of buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM EDTA, 0.5 mM PMSF, and 10 μg/ml leupeptin and aprotinin] and incubated on ice for 10 min. The lysate was spun for 30 s to separate the nuclei and supernatant. For cytosol preparation, the supernatant was further centrifuged at 14,000 rpm for 10 min to remove subcellular debris. For nuclear extraction, the nuclei pellet was resuspended in 100 μl of nuclear extraction buffer [20 mM HEPES (pH 7.9), 0.45 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 0.5 mM PMSF, and 10 μg/ml leupeptin and aprotinin] and incubated for 30 min. Thereafter,
the solution was centrifuged at 14,000 rpm for 10 min, and the supernatant (nuclear extracts) was isolated.

**Clonogenic Survival Assay.** Clonogenic survival was defined as the ability of the cells to maintain their clonogenic capacity and to form colonies. Briefly, after exposure to radiation, cells were trypsinized, counted, and seeded for colony formation in 35-mm dishes at 50–5000 cells/dish. After incubation intervals of 14–21 days, colonies were stained with crystal violet and manually counted. Colonies consisting of $\geq 50$ cells were scored, and 4–10 replicate dishes containing 10–150 colonies/dish were counted for each treatment.

**SLDR and PLDR.** These studies were designed to examine the influence of C225 on radiation damage repair. SLDR was demonstrated with classic split dose radiation design using a $^{137}$Cs irradiator (J. L. Shepherd & Associates, Glendale, CA). Exponentially growing SCC cells received a dose of 3 Gy at time 0 and a second dose of 3 Gy at time points ranging from 2–48 h thereafter. The time 0 point shown in Fig. 4 represents the response to a single dose of 6 Gy. During the time interval between successive 3-Gy fractions, cultures were incubated in the absence (control) or presence of 30 nM C225. After the second radiation exposure, cells were harvested and replated for clonogenic survival analysis as described above. PLDR was demonstrated by single-dose irradiation of confluent cultures, and cell survival was measured by clonogenic assay. The time 0 point shown in the left panel of Fig. 5 represents the survival of cells that were plated immediately after radiation. The remaining data points depict the survival of cells with delayed plating at various time points after their exposure to radiation at time 0.

**Statistical Analysis.** In all experiments, differences among treatment groups were examined by one-way ANOVA or Student’s $t$ test using SAS.

**Results**

**C225 Augments in Vivo Tumor Response of SCC Xenografts to Radiation.** Two human SCC cell lines (SCC-1, SCC-6) were inoculated s.c. into female athymic mice and allowed to grow for 10 days before randomization into four groups. Ten days was the time interval required for xenografts to reach $\sim 20$ mm$^3$ in volume. As shown in Fig. 1, treatment with radiation alone or with C225 alone produced modest inhibition of tumor growth in both SCC-1 and SCC-6 xenografts. In contrast, combined treatment with radiation and C225 produced a marked inhibition in tumor growth over the 55-day observation period in comparison with single modality treatment or control ($P < 0.01$ for all comparisons). In an attempt to examine the *in vivo* interaction of C225 and radiation, low doses of both agents were specifically selected so that their independent effects on tumor growth inhibition would be modest.

We further examined the effect of combining radiation with C225 on the growth response of larger, more well-established SCC xenografts. SCC cells were inoculated as above and allowed to grow until they had achieved a mean volume of 100 mm$^3$ before C225 treatment. As shown in Fig. 2, C225 was then...
administered via i.p. injection on day 23 at a dose of 0.2 mg once a week for 4 consecutive weeks. Radiation fractions were delivered 24 h after each injection of C225 at a dose of 8 Gy. In mice receiving single modality treatment with either radiation or C225, inhibition of tumor growth was observed during the first few weeks after treatment. Thereafter, the tumor growth profile resumed the same growth rate/slope as that of the controls. In contrast, combined treatment with radiation and C225 resulted in dramatic tumor growth inhibition and, in all cases (n = 8 mice), complete tumor regression for up to 100 days of study.

In Vivo Expression of PCNA, VEGF, and FVIII. The expression of several markers of tumor growth and angiogenesis were examined in SCC-1 tumor xenografts. Immunohistochemical staining with PCNA demonstrated the number of proliferating cells to be greatest in the control group, intermediate in the groups receiving single modality treatment with either radiation or C225, and least in the group receiving dual treatment (Fig. 3). Immunostaining for VEGF was far more pronounced in control and radiation groups when compared with groups receiving C225. Using FVIII antibodies directed against endothelial cells, vessel formation was shown to be significantly reduced in the group receiving combined treatment with C225 and radiation compared with that of the remaining groups (Fig. 3). Taken together, these results suggest that C225 may also inhibit tumor angiogenesis in addition to its cell cycle inhibition of proliferation.

SLDR and PLDR. The capacity of C225 to influence SLDR and PLDR was examined in SCC-13Y cells. Fig. 4 depicts the effect of C225 on SLDR after split-dose radiation exposure. Cell survival in control cells shows a steady increase as the time interval between the two radiation fractions increased, which is indicative of SLDR. However, when cells were exposed to C225 during the interval between radiation fractions, cell survival was significantly less (P < 0.05) compared with corresponding controls, which is indicative of SLDR inhibition.

The capacity of C225 to influence PLDR was examined under delayed plating conditions for clonogenic survival. The left panel of Fig. 5 shows the time-dependent response of PLDR in SCC-13Y cells. After a single 9-Gy radiation exposure, delayed plating of control cells between 6–48 h resulted in an increased cell survival compared with that observed with immediate plating. In contrast, treatment with C225 before radiation resulted in a reduced survival (P < 0.02) in comparison with controls. We further examined the influence of C225 on PLDR when varying doses of radiation (e.g., 0, 3, 6, and 9 Gy) were applied, as shown in the right panel of Fig. 5. As expected, control cells showed a greater survival when they were plated 24 h after radiation rather than immediately after radiation. Conversely, in cells treated with C225, cell survival was not increased by delayed plating, suggesting that PLDR was inhibited by C225. Taken together, these results suggest that C225 compromises the capacity of SCCs to accomplish effective repair after radiation-induced damage.

Subcellular Distribution of DNA-PK. The DNA repair enzyme DNA-PK is known to reside primarily in the nucleus where it exerts a major role in repairing double-strand DNA breaks (13, 14). To examine the effect of C225 on the subcellular localization of DNA-PK after radiation, SCC cells were treated with C225 for 1 h followed by radiation. As expected, a major fraction of DNA-PK was localized in the nucleus, and a minor fraction was localized in the cytosol after radiation exposure (Fig. 6). However, dual treatment with radiation and C225 resulted in a readily measurable shift in the DNA-PK distribution ratio, with an increase in the cytosolic level of DNA-PK and a concurrent reduction in the nucleus. The reduc-
tion in the level of DNA-PK in the nucleus suggests a potential mechanism whereby the repair of double-strand DNA after radiation may be impaired by C225.

Kinetics and Cell Cycle Progression. Using flow cytometric analysis, the relative distribution of cells among various cell cycle phases were determined in SCC-13Y cells treated with either PBS, radiation, C225, or the combination of radiation and C225. As shown in Fig. 7, G1 cell cycle arrest was induced in cells exposed to C225 alone, and G2-M cell cycle arrest was induced in cells receiving radiation alone. Dual treatment with radiation and C225 resulted in the accumulation of cells in both the G1 and G2-M phases, with a concurrent reduction of cells within S phase. These results suggest that one potential mechanism whereby C225 may enhance radiosensitivity involves the accumulation of cells in more radiosensitive cell cycle phases, such as G1 and G2-M phases.

Discussion

Previous work has demonstrated C225 to be a potent anti-proliferative agent capable of inhibiting SCC cell growth in culture (6). In addition, C225 enhances in vitro radiosensitivity and promotes radiation-induced apoptosis in SCCs (6). In the present report, we demonstrate that treatment of well-established SCC tumor xenografts with the combination of C225 and radiation can induce complete regression of tumors in athymic mice over a 100-day follow-up period (Fig. 2). The profound antitumor effect observed with combined C225 and radiation (more potent than that observed in cell culture) strongly suggests that mechanisms beyond simple proliferative growth inhibition are operational in the in vivo setting. These mechanisms may include (among others) C225-induced inhibition of DNA damage repair, increased radiosensitivity deriving from specific perturbations in cell cycle phase distribution, enhancement of radiation-induced apoptosis, and inhibition of tumor angiogenesis. From the standpoint of cell line variability, recent evidence also identifies a significant inverse relationship between the magnitude of EGFR expression and both radiocurability and radiation-induced apoptosis (15).

In the present studies, the influence of C225 on radiation damage repair was reflected by the capacity of C225 to inhibit PLDR and SLDR. The precise molecular mechanisms that underlie SLDR and PLDR are not clear. Operationally, SLDR is described as that cellular recovery that occurs during the interfraction interval between split-dose radiation exposure, whereas PLDR is described as that cellular recovery that occurs dependent on postradiation conditions after single-dose exposure (16). Both SLDR and PLDR have been shown to be affected by growth factors (17, 18). For example, bFGF has been shown to
induce PLDR in bovine aortic endothelial cells, and such induction was inhibited by using a neutralizing mAb against bFGF (17). It may be that down-regulation of selected mitogenic signal transduction pathways can inhibit cellular recovery processes after radiation damage, but no precise mechanistic scheme is presently appreciated. A similar postulation has recently been put forth in the HER-2/breast cancer system wherein enhanced radiation response and diminished DNA repair is observed in human MCF-7/HER-2 breast cancer cells after mAb blockade of the HER-2 receptor (19).

Recent evidence has suggested a link between the repair of DNA double-strand breaks and SLDR or PLDR (20). Li et al. (21) reported that severe combined immunodeficiency fibroblasts, which were deficient in repairing DNA double-strand breaks, demonstrated a lack of PLDR. Using two isogenic human malignant glioma cell lines, Allalunis-Turner et al. (22) demonstrated that cell lines lacking the catalytic domain of DNA-PK did not exhibit SLDR. In our studies, C225 induced a redistribution of DNA-PK with a reduction in the level of DNA-PK in the nucleus of SCC cells exposed to radiation. This finding is consistent with a recent report demonstrating a redistribution of DNA-PK from nucleus to cytosol after exposure to C225 as measured by immunohistochemical analysis (11). Therefore, it is possible that the observed inhibitory effects of C225 on PLDR and SLDR were mediated in part by impairing the function or activity of DNA-PK within the nucleus of SCCs.

The general profile of cell cycle perturbation, which results after exposure to radiation, has been well established (23–25). In general, cells damaged by radiation will arrest in the G2-M phase presumably to initiate repair of DNA damage before proceeding (26). However, treatment of tumor cells with C225 induces growth arrest primarily in the G1 phase (10, 27). When tumor cells are simultaneously exposed to modest doses of radiation plus C225 (exposures that individually produced only transient responses in SCC tumor xenografts), profound tumor cell kill is observed, which may reflect a cellular intolerance to concurrent blockade at these two distinct cell cycle checkpoints. Cellular damage induced by radiation signals proliferating cells to pause to initiate repair, and many repair processes require growth factors to proceed effectively. It may be that cells attempting to repair radiation-induced damage, that simultaneously undergo molecular blockade of the EGFR system with agents such as C225, are unable to facilitate effective repair and recovery, thereby contributing to cell death.

The markedly increased antitumor potency of C225 and radiation in vivo (over that observed in vitro) suggests that factors beyond the confines of the clonogenic cell may influence the in vivo response. One possible microenvironmental mechanism involves tumor angiogenesis, which may itself be affected by the functional activity of the EGFR system. Recent findings suggest that the use of an angiogenesis inhibitor (angiostatin) during radiation therapy can significantly enhance response in human tumor xenografts (28). Indeed, several recent associations between the EGFR pathway and tumor angiogenesis have been identified (12, 29, 30). For example, C225 was shown to down-regulate the expression of several angiogenic factors, including VEGF, interleukin 8, and bFGF in A431 and human transitional cell carcinoma of the bladder (12). This down-regulation was postulated to be mediated via reduction of AP-1 activity, which was shared by the promoters of VEGF, bFGF, and interleukin 8. In the present studies, we observed an inhibition of VEGF expression and reduced number of tumor vessels via staining with the endothelial cell marker (FVIII) in xenografts after treatment with C225 and radiation. These findings provide indirect evidence that C225 may also inhibit tumor angiogenesis in addition to the observed effects on radiation-
growth receptor blockade (e.g., herceptin, rituxan), C225 plus radiation therapy in SCC of the H&N represents a promising new molecular cancer therapy approach that has recently commenced formal investigation in Phase III clinical trials.

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References


induced cytotoxicity. Studies to clarify and augment these findings using complementary in vivo assays of angiogenesis are being developed.

In conclusion, epithelial tumors that are rich in their expression of EGFR hold special promise for the receptor blockade approach. SCCs of the H&N are notably robust in their EGFR expression and therefore represent a logical experimental model for EGFR inhibition. In addition, SCCs of the H&N are particularly rapid proliferators, which lends favorably to the anti-proliferative impact of EGFR blockade. The studies presented in this report demonstrate that human H&N cancer cells are particularly sensitive to radiation damage when the EGFR signaling pathway in these cells is blocked by C225. Most impressively, the in vivo tumor response after the combined administration of C225 and radiation is dramatic and long-lasting, as demonstrated within the xenograft model system.

Such profound antitumor activity in vivo appears to derive from not only proliferative growth inhibition (with associated cell cycle redistribution), but also from inhibition of postradiation damage repair and inhibition of tumor angiogenesis.

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