Radiation-induced Enhanced Proliferation of Human Squamous Cancer Cells in Vitro: A Release from Inhibition by Epidermal Growth Factor

Brian D. Kavanagh, Peck-Sun Lin, Phyllip Chen, and Rupert K. Schmidt-Ullrich
Department of Radiation Oncology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0058

ABSTRACT
Ionizing radiation is believed to stimulate the repopulation of squamous carcinoma cells that survive the early portion of a fractionated course of radiotherapy. To characterize any intrinsic radiation-induced adaptive response and to examine whether epidermal growth factor (EGF) influences this response, A431 and 183A cells were irradiated with repeated daily exposures of 0.5–0.75 Gy and then grown in monolayer culture for 7 days with or without EGF at a 1 ng/ml concentration. Cell numbers were quantified using a microtiter dye-reduction assay. EGF alone caused approximately 70% and 30% growth inhibition of human SC A431 and 183A cells, respectively. Although radiation alone did not affect proliferative rates in these conditions, radiation eliminated the EGF-related growth inhibition in both cell lines. This effect was dose dependent in single radiation exposure experiments. Cell cycle analyses indicated that EGF initially promoted entry into S-phase 3 days after treatment but caused a G1-S block after 7 days. Treatment with radiation recruited cells into S-phase and G2-M, an effect which was sustained 7 days after treatment, overriding the influence of EGF. Radiation-induced modulation of the response of human squamous carcinoma cells to EGF in vitro after single and repeated radiation exposures suggests a proliferation response that may underlie enhanced repopulation of tumor clonogens in vivo.

INTRODUCTION
Retrospective analyses of the relationship between tumor control probability of head and neck carcinomas and total treatment time have led to the hypothesis that fractionated radiotherapy induces accelerated repopulation of SC cells that survive the early portion of the treatment course (1, 2). The mechanisms by which repeated radiation exposures might promote repopulation are not completely understood (3). Treatment-related tumor shrinkage may improve in vivo nutrient supply to viable clonogens and thus recruit quiescent cells into active cycling. The spheroid model has yielded important in vitro evidence of this phenomenon, including the demonstration that multiple radiation exposures can select rapidly proliferating cells with correspondingly increased proportions of S-phase cells (4). The potential shortcoming of the spheroid system is the inability to differentiate between nutrient-dependent repopulation and accelerated proliferation as an intrinsic cellular response to radiation exposure. One objective of the present experiments was to study the relative importance of the latter by means of an experimental system of monolayer cultures wherein nutrient supply is not a limiting factor.

A monolayer system is also well suited to establish conditions for studies of growth factor effects on cellular proliferation and radiation responses. Many studies on the expression regulation of EGF and its principal receptor, EGF-R, have been conducted with the human vulvar SC cell line A431. The A431 cells express high levels of EGF-R, and treatment with exogeneous EGF at concentrations on the order of 1 ng/ml or more characteristically inhibits proliferation (5–9). The 183A cell line, derived from a SC of the head and neck, has likewise been characterized with respect to EGF-R expression (10) and growth factor response (11). Because of the background information, these two cell lines are useful for examining the interactions of EGF and radiation with regard to cellular proliferative responses. We suspect that any intrinsic adaptive cellular response that influences proliferation would involve effects on important growth factor receptor-ligand interactions. The second, more specific objective of the present set of experiments was to examine whether radiation can generate a functional change in response to EGF such that a growth inhibitory effect might be counteracted. Such a process might influence repopulation during multifraction clinical radiotherapy.

MATERIALS AND METHODS
Reagents and Cell Lines. Recombinant human EGF was obtained from Oncogene Science (catalog no. PF011–100; Uniondale, NY). The A431 cells were originally obtained from American Type Culture Collection (Rockville, MD). The 183A cells were provided by Dr. Peter Sacks (Memorial Sloan-Kettering Cancer Center, New York, NY). Stocks of both cell lines were maintained at 37°C in 95% air-5% CO2, in monolayer culture in RPMI 1640 media (Sigma Chemical Co., St. Louis, MO) supplemented with 5% FCS (HyClone Laboratories, Logan, UT), which served as the control medium in all experiments (RPMI/5% FCS). Cells were tested to be free of Mycoplasma.

Irradiation Techniques. Cells were irradiated using an 60Co unit at a dose rate of 1 cGy/s. Cells were maintained in RPMI/5% FCS during all radiation exposures, which were per-
formed at room temperature. Dosimetry was verified by the placement of thermoluminescent dosimeters on the 96-well plates and T-25 flasks to represent the actual treatment conditions.

**Cell Growth Assay.** To quantify the total number of cells surviving irradiation, a colorimetric microtiter (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay of tetrazolium salt reduction was used (12). The measured absorbance was linearly proportional to the number of cells in the well over the absorbance range of 0.2–1.5. The Student’s t test was used to compare results.

**Steady-State Growth Conditions.** To establish a dose range in which the effect of radiation cytotoxicity was counteracted equally by cellular proliferation, A431 cells in RPMI/5% FCS were irradiated with daily exposures of 0–2 Gy for a 3-week period. Initial inocula were 1000 cells/well of each 96-well plate. Medium was replenished weekly.

**Repeated and Single Exposure Irradiation Experiments.** Cells in monolayer culture were irradiated with either repeated daily exposures (range, 0–2 Gy) or a single radiation exposure (range, 0–5 Gy). After irradiation, the cells were grown in RPMI/5% FCS with or without EGF for 7 days, at which time cell numbers were quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Experiments using A431 and 183A cells were conducted under identical conditions. For the repeated exposure experiments, 1000 cells/well were plated of a 96-well plate in 0.2 ml RPMI/5% FCS. One day later, the cells received the first of three daily radiation exposures of 0.5 or 0.75 Gy. After the third exposure, the medium was replaced with fresh RPMI/5% FCS with or without EGF at a 1 ng/ml concentration, and the number of cells in each well was quantified after 7 days. For the single exposure experiments, cells were plated in a similar manner and irradiated with doses up to 5 Gy. After 24 h, the medium was replaced with RPMI/5% FCS or medium containing EGF at 1 or 10 ng/ml concentrations. The nonirradiated controls received fresh medium 1 day after initial plating, RPMI/5% FCS without or with EGF at the appropriate concentrations, and cell numbers were determined after 7 days.

**Cell Cycle Analyses.** To examine the effect of repeated radiation exposures and EGF on cell cycle distribution, A431 cells were irradiated, grown in RPMI/5% FCS with or without EGF at a 1 ng/ml concentration, and then analyzed by flow cytometry. Subconfluent A431 cells in T-25 flasks were ex-

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**Fig. 1** Proliferation of A431 cells after repeated daily exposures of 0–2 Gy. Absorbance is proportional to cell number.
posed to 0.75 Gy daily for 3 days. After the last exposure, the media were replaced with fresh RPMI/5% FCS with or without EGF at 1 ng/ml concentration. On days 3 and 7 after the last radiation exposure, cells were harvested, fixed in 70% ethanol, and stained with propidium iodide. The DNA content was analyzed with a fluorescence-activated cell sorter (EPICS 753; Coulter Electronics, Miami, FL) using an established program to compute the relative distribution of cells in the different phases of the cell cycle (13).

RESULTS

Baseline Growth Conditions. Fig. 1 illustrates cell proliferation over a 3-week period. Cells receiving daily exposures of 0 or 0.25 Gy outgrew the cytotoxicity of radiation and reached a plateau value of an absorbance of about 2. Cells numbers after 0.5 or 0.75 Gy remained approximately constant for the duration of the experiment, whereas cells receiving 1 or 2 Gy declined in number and eventually fell to levels beneath the range of accurate quantification.

Repeated Exposure Experiment. Fig. 2 illustrates the proportionate cell number of A431 and 183A cells after 1 week of growth in media with EGF normalized to growth in control media. Each result is an average ± SD of at least six experiments. Measurement of cell numbers on day 2 and 7 allowed for calculation of the average cell doubling time under control conditions to be 2.6 days for A431 cells and 2.7 days for 183A cells during that period (data not shown). Both cell lines demonstrate growth inhibition by EGF, approximately 70% for A431 and 30% for 183A cells.

Because the initially established baseline growth conditions for A431 cells indicated that the toxicity of a daily exposure of 0.5–0.75 Gy was equally counteracted by cell proliferation over a 3-week period (Fig. 1), it was decided to proceed with a short course of repeated exposures within this range to analyze the proliferation rate within the population of cells surviving the treatment. The number of viable cells at completion of the exposures would be expected to be approximately equal to the number at the start. Since 183A cells grew at the same rate as A431 cells in the control conditions, the same radiation doses were used for both cell lines. Because there was no significant difference between cells pretreated with exposures of 0.5 or 0.75 Gy, the results have been combined for clarity. Cell numbers in the 7-day growth assays for A431 and 183A cells after irradiation alone were not significantly different from nonirradiated controls (Fig. 2). Likewise, calculated doubling times based on comparisons of cell number at days 2 and 7 were not significantly different from those of controls (data not shown). Also depicted in Fig. 2 is 7-day growth in media with EGF after three daily radiation exposures of 0.5 or 0.75 Gy. Radiation exposure modulates the response of both cell lines by eliminating the growth inhibitory effect of EGF. Relative to EGF alone, repeated radiation exposures accelerated the proliferation of both cell lines with \( P \) values of 0.01 and 0.001 for 183A and A431 cells, respectively.

Single Exposure Dose Response. Since a total dose as little as 1.5 Gy in three exposures was observed to affect the proliferative response to EGF, single exposure experiments were performed in an effort to identify the pattern of dose response with a smaller total radiation dose. Fig. 3 illustrates growth in media with EGF as a percentage of growth in control media after single radiation exposures of 0–5 Gy. The data points are averages ± SD from at least three experiments, each involving assays of 8–12 wells. In both cell lines, irradiation limits EGF-related growth inhibition in a dose-dependent manner. There is a suggestion of a sigmoidal pattern for A431 cells at the 1 ng/ml concentration level (Fig. 3a), whereas for 183A cells the lesser initial EGF effect in these conditions does not allow graphic discrimination of a similar pattern (Fig. 3b). A higher concentration of EGF was also tested to determine whether stronger growth inhibition might allow better discrimination of the dose-response relationship. However, in both cell

Fig. 2 Growth of A431 and 183A cells with EGF at a concentration of 1 ng/ml, relative to growth without epidermal growth factor (control), with or without preirradiation (radiation). The radiation schedule included three daily exposures of 0.5–0.75 Gy. ■ control; □, EGF; △, radiation; ■, irradiation + EGF.
lines, EGF at 10 ng/ml exerts an overriding inhibitory effect which is only minimally affected by radiation (Fig. 3).

Cell Cycle Analyses. The control cells were found to have the following cell cycle distribution at 3 days after plating (mean ± SD): G0-G1, 64 ± 8%; S-phase, 25 ± 4.5%; and G2-M, 11 ± 3.5%. Fig. 4a depicts the percentage of change of G0-G1, S-phase, and G2-M after growth with EGF at a 1 ng/ml concentration (EGF), after irradiation alone (radiation), and after irradiation followed by EGF exposure (radiation + EGF). The control cells had the following cell cycle distribution at 7 days after plating: G0-G1, 63 ± 8%; S-phase, 26 ± 10%; and G2-M, 11 ± 2%. Fig. 4b shows the percentage of change from these values for the three conditions EGF, radiation, and radiation + EGF. There is an increase of S-phase and G2-M at 3 days after EGF, radiation, and radiation + EGF with P values < 0.05 in each case. After 7 days of EGF treatment, the effect is diminished, and the decrease in the S-phase fraction suggests a G1-S block. However, the significant increase of S-phase and G2-M is sustained 7 days after radiation, and it is not affected when EGF is added following radiation. Although the EGF-induced early shift into S-phase and G2-M followed by a G1-S block suggests the possibility of a biphasic growth pattern, within our experimental conditions only monophasic exponential growth was observed from day 1 through day 7 (data not shown).

DISCUSSION

The present experiments demonstrate that in A431 cells and 183A cells single radiation exposures up to 5 Gy and repeated exposures of 0.5 or 0.75 Gy counteracted a growth inhibitory effect of EGF at 1 ng/ml concentration. The effect

Fig. 3 Growth with EGF (1 ng/ml and 10 ng/ml) expressed as a proportion of growth without EGF after single radiation exposure of 0–5 Gy for A431 cells (a) and 183A cells (b).
was radiation dose dependent for single exposure experiments with an almost complete reversal of EGF-induced growth inhibition after 1 Gy. Cell cycle analyses after repeated radiation exposures demonstrated a decreased proportion of cells in the G$_0$-G$_1$ with a proportional increase in G$_2$-M. These findings are compatible with an increased proportion of actively cycling cells.

Many carcinoma cells are growth regulated through autocrine growth factor/growth factor receptor circuits. EGF acting through EGF-R is one of the dominant systems in SC cells. Single radiation exposures as small as 1 Gy can affect expression of EGF-R in A431 cells, and it has been suggested that this effect represents an adaptive response which primes cells for rapid repopulation (14). Similar findings of radiation-induced up-regulation of EGF-R and transforming growth factor α have been reported for MCF-7 human mammary carcinoma cells (15, 16). Within the present studies, it is at first difficult to reconcile the possibility of increased surface expression of a receptor for an inhibitory growth factor with an observation of improved cell proliferation with the exogenous administration of the same growth factor. One possible explanation is that the transiently observed alteration of receptor expression represents an interruption of the typically rapid receptor internalization and recycling process, the rate of which has been observed to be dependent on cell density and EGF concentration (17). Additional studies are presently underway to characterize the effect of radiation on the process of receptor recycling.

In MDA-468 cells, which express high levels of EGF-R similar to A431 cells, one mechanism of EGF-induced growth
inhibition results from a reversible cell cycle block at the G₁-S (18). At the 1 ng/ml concentration used in our experiments, EGF promoted entry into S-phase and G₂-M at 3 days (Fig. 4a), but longer exposures reversed that early trend to an apparent decrease in the S-phase fraction (Fig. 4b) at 7 days, consistent with a G₁-S block. This pattern is qualitatively similar to the observations of cell cycle effects of EGF on hormone-deprived MCF-7 cells in which an early promotion into S-phase followed by a return to preexposure levels (19). In A431 cells both radiation and EGF can cause a G₁ arrest, and high concentrations of EGF (50 ng/ml) may enhance a radiation-induced G₂-M arrest (20). In the present study, there was not a preferential increase of S-phase relative to G₂-M after irradiation (Fig. 4), thus no direct evidence of arrest in G₂-M at 7 days. We interpret the sustained increases of S-phase and G₂-M as recruitment of cells into active proliferation. Most importantly in the present studies, the effect of the radiation is overriding that of EGF such that recruitment into S-phase and G₂-M is sustained in the presence of EGF (Fig. 4b).

Whereas the present work involves radiation-induced modulation of the proliferative response to EGF, other studies of the interactions of radiation and EGF have differed in both methodology and experimental design. Clonogenic survival assays have been used to investigate EGF-R-dependent radiosensitization by EGF. Treatment of irradiated A431 cells with a 10 ng/ml concentration of EGF lowers plating efficiency compared with irradiated controls without EGF, an effect which is recognized to be entirely separate from the EGF-related inhibition of proliferation of nonirradiated, previously plated cells (9). The impact of radiation on the latter effect would not be easily observed by clonogenic assay, since EGF would be expected to affect proliferation rates rather than clonogenicity (21). Interestingly, preconditioning cells by growing them in media with 50 ng/ml EGF can remove the EGF-related growth inhibition, similarly to the effects of irradiation in the present experiments (9).

The present study establishes conditions in which clinically relevant doses of radiation promote cellular proliferation by eliminating EGF-related growth suppression. This particular mechanism of radiation-induced enhancement of proliferation in the presence of EGF might be active in vivo during fractionated radiotherapy and contribute to accelerated proliferation of viable tumor clonogens.

REFERENCES

Radiation-induced enhanced proliferation of human squamous cancer cells in vitro: a release from inhibition by epidermal growth factor.

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