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Radiation-resistant and repair-proficient human tumor cells may be associated with radiotherapy failure in head- and neck-cancer patients

(inherent radioresistance/DNA repair/predictive assay)

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ABSTRACT **Inherent cellular radioresistance and repair of x-ray damage was studied in 19 early-passage squamous cell carcinoma lines derived from head- and neck-cancer patients with known clinical results following radiotherapy. Human tumor cells that were radioresistant and/or proficient in accumulation/repair of x-ray damage were cultured from patients unsuccessfully treated with radiotherapy. Thus, the presence of radiation-resistant and repair-proficient tumor cells was associated with clinical radiation failure, suggesting the possibility of a predictive assay based on *in vitro* radiobiological parameters.**

Radiation therapy is a central component of modern cancer treatment. Identification of patients most likely to benefit from treatment with ionizing radiation, as well as of basic mechanisms of radiotherapeutic failure, might improve the therapeutic ratio (1). Therefore, the study of inherent cellular radioresistance as well as the ability to accumulate and repair x-ray damage in individual human tumor cells has received increasing interest in recent years. Most data on the radiobiological parameters of human tumor cell culture are derived from established cell lines, and rarely is the clinical response of the patients to radiotherapy correlated with *in vitro* biological parameters (2–6).

The accumulation and repair of sublethal damage induced by ionizing radiation has been postulated to be important in clinical radiotherapy (7). When a single dose of x-rays is divided into two fractions separated by an interval of several hours, an enhancement in survival occurs. This recovery phenomenon has been interpreted as reflecting the repair of sublethal damage induced by the first dose in cells that survive this dose (8). The magnitude of this effect can be expressed by the extrapolation number, $\bar{n}$, which is the back-extrapolation of the slope to the ordinate (a measure of the extent of the shoulder region of the survival curve). The shoulder is thought to represent the ability of cells to accumulate and repair sublethal x-ray injury (8). The repair of potentially lethal damage has also been postulated to be an important repair process in clinical radiotherapy. Potentially lethal damage is operationally defined as damage that, if unrepaired, is lethal (9). Plateau-phase cultures represent *in vitro* systems that have certain characteristics of tumors *in vivo*, including a large proportion of nonproliferating G_1-phase cells (10). We specifically define the repair of potentially lethal damage as the enhancement in survival following a delay in subculture of cells to low density from plateau-phase cultures after treatment with ionizing radiation. This process may be analogous to liquid holding recovery in bacteria and yeast (11, 12).

To determine the contributions of cellular radioresistance, accumulation of sublethal damage, and repair of potentially lethal damage to clinical outcome, we investigated these parameters in 19 early-passage human squamous cell carcinoma lines derived from patients with squamous cell carcinoma of the head and neck with known clinical results following radiotherapy. Cell populations from each tumor were serially cultivated under identical conditions and were studied between 10 and 15 passages after explant and correlated with radiocurability (local control). We reported several of these tumor cell lines in a preliminary communication (13) but were unable to correlate radiobiological parameters with clinical outcome because too few cell lines from patients were available for analysis.

MATERIALS AND METHODS

Isolation of Tumor Cells. Methods of establishment and characterization of squamous cell carcinoma lines have been published and are briefly summarized here (14, 15). Biopsy specimens of squamous cell carcinoma were obtained from patients seen in the multidisciplinary head and neck tumor clinic at the Dana–Farber Cancer Institute and the Joint Center for Radiation Therapy. Culture conditions and procedures were similar to those for preparing keratinocyte cultures from normal skin, including coculture with a 3T3 fibroblast feeder layer (14). Biopsy samples were placed into culture within 2 hr of removal. Samples were rinsed with serum-free medium containing penicillin or streptomycin and cut into pieces 3 mm in diameter. A portion was sectioned and stained with hematoxylin and eosin in order to confirm that the biopsies contained squamous cell carcinoma. The remaining fragments were minced with scissors into pieces <1 mm in diameter and were distributed to culture dishes and held to the surface with a small plasma clot. One day after plating, mitomycin C-treated, Swiss mouse embryonic 3T3 fibroblasts were added as a feeder layer. The cultures were maintained at 37°C in a humidified atmosphere at 5% CO_2 in air.

Growth medium consisted of Dulbecco's modified Eagle's medium, 20% fetal bovine serum, and hydrocortisone (0.4 μg/ml). Primary cultures were subcultured after 1–2 weeks, at which time individual explant colonies had attained a diameter of 0.5–1.0 cm, and before neighboring colonies had merged to make a confluent monolayer. Tumor cell populations were disaggregated by a 15- to 30-min incubation with 0.05% trypsin plus 0.02% EDTA at 37°C and were serially

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Abbreviation: PLDR, potentially lethal damage repair.

passaged at 1–3 × 10^4 cells per 60-mm dish together with 3T3 feeder cells. Each passage was equivalent to about 7–10 cell generations. As reported previously (15), the tumor lines retained unique aneuploid karyotypes and distinctive morphological characteristics indefinitely from the first passage, suggesting that the lines represent a major stem cell population of their respective tumors.

Radiation Experiments. X-ray survival curves were determined as follows. Cells at the 10th to 15th passages were maintained in medium without 3T3 cells at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were trypsinized (0.05% trypsin) from stock cultures and between 500 and 40,000 cells were plated in 100-mm-diameter dishes and allowed to enter exponential growth. Irradiation was carried out 18 hr later with a GE Maximar x-ray generator at 220 kV peak and 15 mA, yielding a dose rate of 80 rads/min (1 rad = 0.01 Gy). Immediately after irradiation, the cultures were returned to the incubator. After 18–24 days, the cells were fixed and stained with crystal violet. Only colonies of >50 cells were scored as survivors. All data points are the results of 2–4 experiments. Radiation survival curve parameters measured as the D_0, which is the inverse of the slope of the radiation survival curve, and the extrapolation number, $\bar{n}$, which is the back-extrapolation of the slope to the ordinate. These parameters were determined by a least-squares regression analysis of all data points.

Studies of potentially lethal damage repair (PLDR) were performed as follows. Cells were initially seeded into 60-mm plastic tissue culture dishes and grown to confluency. Culture medium was renewed daily for 3 days and experiments were performed on day 4. Cells were irradiated at room temperature and afterward were returned to the incubator. Single dishes were removed and cells were subcultured and seeded at low density (10,000–80,000 cells per 100-mm dish) at regular intervals thereafter.

A dose of 700 rads was used to study PLDR. Initial (0-hr subculture) surviving fractions were similar in 17 of the 19 cell lines. Lines SCC-61 and SCC-73 were exceptions and showed lower initial surviving fractions than the other cell lines studied. The enhancement in survival, as measured by the factor of increased colony-forming ability resulting from delay in subculture after irradiation, is interpreted as being due to the repair of potentially lethal damage. PLDR is expressed in terms of enhancement in surviving fraction as a function of time interval between radiation and subculture after a single dose of radiation and is expressed as the recovery ratio (R/R_0) by dividing the 24-hr surviving fraction (R) by the 0-hr surviving fraction (R_0). Although growth of some of the cell lines was greatly enhanced by the use of feeder-layer support, survival-curve parameters were independent of the presence of a feeder layer. Feeder layers used in radiation experiments were rendered nonproliferative with 10,000 rads from a 2 Ci (1 Ci = 37 GBq) cobalt-60 source.

RESULTS

Table 1 shows a summary of clinical-state disease site and response to radiotherapy (and other treatment) in patients whose biopsy samples were derived from local recurrences following radiotherapy. All patients had completed a course of radiation therapy undertaken with curative intent. Portal films and/or charts were reviewed to certify that tumors were in-field failures and not marginal recurrences or the result of technical errors or excessively protracted fractionation of the radiotherapy. (Fractionation refers to the division of a dose of radiation into multiple treatments, usually delivered daily over a period of time.) Biopsy samples were obtained from sites where radiation failures occurred. Cell lines SCC-25 and SCC-76 were especially interesting because the tumors from which these cells were derived increased in size during the interval that patients were being treated with radiation. This is extremely unusual. Table 2 shows $\bar{n}$, D_0, plating efficiency, 0- and 24-hr surviving fractions (R_0 and R), and 24-hr recovery ratio (PLDR) in tumor cells derived from patients who suffered local radiation failures. The 24-hour recovery ratio (R/R_0) represents the amount of PLDR performed by each

Table 1. Cell lines derived from tumors after radiotherapy

Line	Stage*	Site	Radiotherapy and response†	Comments
SCC-4	T_3N_0	Floor of mouth	Little response; persistent tumor	In-field persistence
SCC-25	T_2N_1	Oral (tongue)	Grew during radiotherapy	Unusually aggressive tumor
			Treatment aborted at 3400 rads	Patient treated with 200 rads twice a day with no effect on rapidly increasing size
SCC-35	T_4N_0	Pyriform sinus	Complete response	In-field recurrence biopsied 2 years later
SCC-13	T_2N_0	Skin of face	Complete response	Recurrence 5 months later
SCC-49	T_2N_0	Tonsil	Partial response	In-field recurrence 14 months later
SQ-50	T_4N_2	Supraglottic larynx	Partial response	Preoperative treatment 4000 rads; postoperative treatment 2000 rads. Persistent disease
SCC-76	T_4N_0	Maxillary antrum	Postoperative radiation treatment following antrectomy	Partial response to preoperative chemotherapy; unusually aggressive tumor
			Tumor grew during radiotherapy	
			Treatment terminated at 4856 rads	
SQ-9G	T_3N_1	Tonsil	Complete response to definitive radiation therapy (6800 rads/7 weeks)	Primary radiation therapy failure after 7 months
SQ-20B	T_2N_0	Larynx	Complete response to primary radiotherapy (6558 rads/6½ weeks)	Failure after 4 months
SQ-31	T_2N_0	Pyriform sinus	6740 rads/7 weeks	Failure to radiation therapy/chemotherapy at 7 months
			Continued complete resolution of tumor following complete response to chemotherapy	
SQ-43	T_1N_0	Supraglottic larynx	6500 rads/6½ weeks	Radiotherapy followed by laryngectomy
			Complete response?	Biopsy of a stomal recurrence 7 months following radiotherapy

*TNM system conforms to the American Joint Committee for Cancer Staging and End Result Reporting (21).
†Partial response is defined as a decrease in the tumor diameter by at least 50%; complete response is defined as complete disappearance of tumor.

Table 2. Radiobiological parameters of cell lines derived from tumors after radiotherapy

Line	$\bar{n}$	D_0, rads	0-hr surviving fraction (R_0)	24-hr surviving fraction (R)	PLDR (R/R_0)	Plating efficiency, %
SCC-4	1.49	169	0.012	0.053	4.4	8.5–15.2
SCC-25	1.53	142	0.010	0.064	6.7	7.2–17.8
SCC-35	1.63	184	0.089	0.122	1.4	21.6–55.7
SCC-13	2.11	128	0.014	0.029	2.2	13.8–19.1
SCC-49	1.55	170	0.011	0.052	4.9	11.7–17.2
SCC-50	1.34	179	0.008	0.024	3.0	14.5–37.6
SCC-76	1.47	197	0.062	0.182	2.9	18.9–24.7
SQ-9G	1.44	146	0.019	0.035	1.9	7.6–23.0
SQ-20B	1.40	239	0.087	0.174	2.0	30.5–47.1
SQ-31	1.36	226	0.044	0.109	2.5	3.5–20.0
SQ-43	1.99	146	0.015	0.035	2.4	3.2–52.2
(Mean ± SEM)	1.57 ± 0.08	175.1 ± 10.6	0.034 ± 0.01	0.080 ± 0.017	3.1 ± 0.05	

Numbers shown were derived from three experiments.

cell line. D_0 (radiosensitivity) values ranged from 128 to 239 rads (mean 175.1 rads). Extrapolation numbers ($\bar{n}$) ranged from 1.34 to 2.11 (mean 1.57). The standard error of the mean $\bar{n}$ ranged from 0.01 to 0.49 for individual tumor cell line determinations. The surviving fraction after 24-hr recovery ranged from 0.024 to 0.182 (mean 0.080). PLDR ranged from 1.4 to 6.7 (mean 3.1).

Table 3 shows a summary of clinical stage, tumor site, and response to radiotherapy (and other treatment) as well as local control (radiocurability) in patients who had a biopsy prior to radiotherapy undertaken with curative intent. Three patients had chemotherapy and three patients had surgery prior to the initiation of radiotherapy. One patient had both preoperative and postoperative radiotherapy. Local-control results of all patients were assessed in the multidisciplinary Dana–Farber Cancer Institute/Joint Center for Radiation Therapy head-and-neck clinic, and recurrent tumors were proven by biopsy. One patient died of a myocardial infarction and showed no histological evidence of tumor at autopsy.

Table 4 shows a summary of $\bar{n}$, D_0 (radiosensitivity), plating efficiency, 0- and 24-hr surviving fraction, and 24-hr recovery ratio (PLDR) in cells derived from tumors biopsied before initiation of treatment. D_0 values ranged from 107 to 213 rads (mean 146.3 rads), and $\bar{n}$ ranged from 1.02 to 1.83 (mean 1.55). The 24-hr recovery ratio (PLDR) ranged from 2.0 to 20.3 (mean 7.2). The standard error of the mean $\bar{n}$ ranged from 0.02 to 0.43 for individual tumor cell line determinations.

Fig. 1 shows representative survival points of five highly radioresistant tumor cell lines ($D_0 \geq 184$) when compared to the distribution of radiosensitivity seen in normal human fibroblasts (16).

DISCUSSION

Among tumor cells associated with radiation failure (Table 2 and patients SCC-61, SCC-71, SCC-66, and SQ-38), lines SQ-31, SQ-20B, SCC-76, and SCC-35 were highly radioresistant (D_0 184–239 rads) when compared to human diploid cell strains (16, 17). These early-passage human squamous cell carcinoma lines are within the range of radioresistant (RAD^+) human tumor cell lines defined in our laboratory (18). Cell lines SCC-50, SCC-49, and SCC-4 were also radioresistant (D_0 169–179 rads). Cell lines SQ-20B and SQ-31 were derived from early clinical stage (<3-cm) lesions. Small tumor size correlates with a high local control rate following radiotherapy; however, both of these patients failed radiation treatment. The tumor cell lines derived from these patients were the most radioresistant in our study (D_0 226 and 239 rads, respectively). These data suggest that the presence of radioresistant tumor cells accounted for these treatment failures and may be a biological determinant of radiocurability.

A clearly radioresistant cell line associated with treatment success, SQ-39 (Table 4), was derived from a patient who underwent a resection before postoperative radiotherapy was given. The radioresistant tumor cell population may have been removed or sufficiently reduced so that the radiation

Table 3. Human tumor cell lines derived before initiation of radiotherapy

Line	Stage	Site	Radiotherapy and response	Comments
SCC-9	T_2N_1	Oral (tongue)	Preoperative 4500 rads; postoperative 2000 rads	No tumor in surgical specimen Died of distant metastasis
			Complete response to radiotherapy prior to surgery	Local control Free of local disease at 26 months
SCC-61	$T_{4X}N_{2BX}$	Oral (tongue)	Partial response with almost complete regression after change in fractionation	Unusually aggressive tumor that enlarged on standard fractionation treatment. Changed to 100 rads three times a day
SCC-73	T_4N_0	Retromolar trigone	Postoperative 6840 rads	Good partial response to chemotherapy prior to radiation therapy; local and distant control Died of myocardial infarction; no tumor at autopsy. Free of local disease at 15 months
SCC-71	T_4N_1	Soft palate	Some response but persistent disease	Persistent disease
SCC-66	$T_{4X}N_0$	Floor of mouth	Partial response to radiation therapy, but never disease-free	Persistent disease
SQ-29	T_3N_1	Retromolar trigone	Postoperative treatment 6000 rads following composite resection	Partial response to chemotherapy prior to surgery/radiation therapy No evidence of disease at followup time (25 months)
SQ-38	T_3N_0	Retromolar trigone	External beam 5000 rads; gold seed implant 3000 rads (total 8000 rads) Questionable whether tumor was completely cleared	Recurrence at 6 months
SQ-39	T_3N_{2A}	Retromolar trigone	Postoperative 6000 rads	Partial response to chemotherapy prior to surgery. No evidence of disease at follow-up time (23 months)

Table 4. Radiobiological parameters of cell lines derived from tumors before initiation of radiotherapy

Line	$\bar{n}$	D_0, rads	0-hr surviving fraction (R_0)	24-hr surviving fraction (R)	PLDR (R/R_0)	Plating efficiency, %
SCC-9	1.39	134	0.015	0.077	5.1	4.9–12.9
SCC-61	1.83	107	0.002	0.030	20.3	6.0–18.3
SCC-73	1.17	108	0.005	0.045	9.3	3.6–12.0
SCC-71	1.45	160	0.018	0.058	3.2	4.0–36.8
SCC-66	2.14	129	0.027	0.068	2.5	0.6–14.3
SQ-29	1.55	173	0.042	0.082	2.0	1.6–15.7
SQ-38	1.82	146	0.008	0.101	12.5	19.4–25.8
SQ-39	1.02	213	0.017	0.048	2.8	4.9–17.1
(Mean ± SEM)	1.55 ± 0.13	146.3 ± 12.5	0.017 ± 0.005	0.064 ± 0.008	7.2 ± 2.3	

Numbers shown were derived from three experiments.

dose delivered achieved a cure. Line SQ-29 was also radioresistant when compared to normal fibroblasts (16, 17). This patient was successfully treated but, like the patient from whom SQ-39 was derived, underwent a surgical procedure followed by postoperative radiotherapy.

In eight patients where biopsy was done before radiotherapy (Table 4), a significant difference ($P = 0.035$) in $\bar{n}$ was found between patients who suffered local recurrence (SCC-61, SCC-71, SCC-66, and SCC-38) and patients who were successfully treated (SCC-9, SCC-73, SQ-29, and SQ-39). No significant differences were found between these two groups with respect to PLDR or 24-hr surviving fraction after treatment (700 rads) of plateau phase cultures; however, our sample size is extremely small. It is intriguing that among biopsy specimens obtained from postirradiation treatment failures (Table 2), tumor cell lines SQ-43 and SCC-13 were derived from early stage (small) tumors, and the $\bar{n}$ values of these cell lines were the largest in our series. Accumulation of sublethal x-ray injury may be an additional biological determinant of radiocurability. This may be especially true in a multifractionated treatment regimen resulting in exponential magnification of the shoulder region of the survival curve.

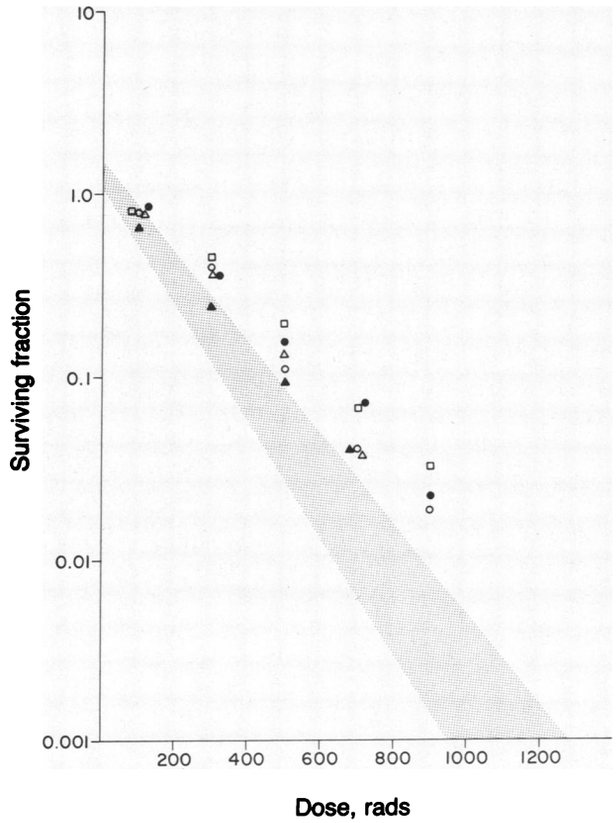

FIG. 1. Representative survival points of five highly radioresistant ($D_0 \geq 184$) cell lines: SCC-35 (○), SCC-76 (△), SQ-20B (□), SQ-31 (●), and SQ-39 (▲). Stippled area is the distribution of radiosensitivity seen in normal human fibroblasts.

Lines SCC-9, SCC-61, SQ-38, and SCC-73 (Table 4), as well as lines SCC-25 and SCC-49 (Table 2), were proficient in the repair of potentially lethal x-ray damage. Lines SQ-38, SCC-61, SCC-25, and SCC-49 were associated with radiotherapy failure. Lines SCC-9 and SCC-73 were associated with successful treatment. The 24-hr surviving fraction after 700 rads in plateau-phase cultures is a function of initial damage induced (D_0 and $\bar{n}$) and repair over a 24-hr period (PLDR). In a previous report, it was suggested that cell lines with surviving fractions >0.1 following 700 rad and 24 hr PLDR time represented histological tumor subtypes associated with a high likelihood of failure of radiation therapy (18). In the present study, cell lines SCC-35, SCC-76, SQ-20B, SQ-31, and SQ-38 all had 24-hr surviving fractions ≥0.1 and were associated with unsuccessful local treatment. Thus, the 24-hr surviving fraction or maximal recovery potential of a tumor cell in plateau-phase cultures may predict radiotherapy failure. Since the PLDR recovery ratio in plateau-phase cultures is a function of initial damage induced (0-hr surviving fraction), the effect of PLDR may be most important in tumor cells of intermediate radiosensitivity. We have previously commented that PLDR may not be expressed under all conditions *in vivo*, since fractionated radiation may induce tumor stem cell repopulation and subsequent proliferation that may fix potentially lethal damage (13) (perhaps analogous to 0-hour subculture *in vitro*). Therefore, PLDR seen *in vitro* may not necessarily be expressed *in vivo*.

In a previous study of established tumor cell lines, radioresistant human tumor cells (RAD^+) were observed, although the differences in radiosensitivity were not as distinct as those in bacterial and yeast systems (18). We suggested that radioresistant and/or repair-proficient human tumor cell lines were derived from tumor cell phenotypes associated with a high probability of radiation failure when treated with standard radiation therapy treatment regimens. In this report, we correlate the presence of radioresistant and repair-proficient tumor cells with clinical failure in head- and neck-cancer patients. The presence of chemoresistant cells has been associated with treatment failures following chemotherapy (19, 20). All tumors in the present study were of the same region (head and neck) and a similar histologic subtype (squamous). A larger clinical study will be necessary to confirm our results, but it seems likely that predictive assays in radiotherapy based on clonogenic survival are possible. Advances in cellular and molecular biology may make rapid identification of radioresistant or repair-proficient tumor cells possible.

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