

Long-term changes in the properties of skin-derived fibroblasts following irradiation of the head and neck

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Abstract. The tumor stroma performs an important role in carcinogenesis. It predominantly consists of fibroblasts and the connective tissue produced by them, and undergoes a multitude of interactions with the surrounding cancer cells. Since irradiation is part of the majority of therapeutic strategies for head and neck squamous cell carcinoma, more information regarding the effects of a previous irradiation on the tumor stroma is desirable. In the present study, fibroblasts were cultivated from human non-irradiated and pre-irradiated skin of the neck for 48 h. Subsequently, analyses of cell viability, apoptosis, necrosis and motility were conducted via MTT assay, Annexin V/propidium iodide staining, electronic cell counting for 4 consecutive days, and scratch assay. Pre-irradiated fibroblasts exhibited a significantly slower growth rate as well as increased rates of apoptosis and necrosis. They also exhibited significantly decreased motility compared with non-irradiated fibroblasts. These results indicated the long-term effects of irradiation on fibroblasts, which may affect cancer recurrence in the irradiated region via the tumor stroma. More information, such as that regarding the secretory capacities of pre-irradiated fibroblasts, is required to evaluate the possible therapeutic implications of these findings.

Introduction

Cell biology and genetic studies indicate that tumor growth is not just determined by malignant cancer cells themselves, but also by the tumor stroma (1). Fibroblasts are non-vascular, non-inflammatory and non-epithelial cells of the connective tissue, and are the principal cellular component of the tumor stroma (2). They are embedded within the fibrillar matrix of the connective tissue and are, to a large extent, responsible for

its synthesis (2). It is becoming increasingly evident that fibroblasts are also prominent modifiers of cancer progression (3,4). There is evidence that a subpopulation of fibroblasts, termed cancer-associated fibroblasts (CAFs), are important promoters of tumor growth and progression (5). CAFs may induce epithelial-mesenchymal transition in epithelial tumor cells, a key factor in the invasion of squamous cell carcinoma (6).

The role of the tumor stroma on cancer progression has also been investigated for head and neck squamous cell carcinoma (HNSCC). HNSCCs are among the most common malignancies worldwide (7). In the USA, it is estimated that ~500,000 new cases of HNSCC are diagnosed per year, equating to an incidence of 14 per 100,000 inhabitants (8). Despite the implementation of multi-modal treatment strategies, including surgery, radiation and chemotherapy, the survival rates have not improved significantly over the past several decades (9). For HNSCC, radiation is part of the majority of therapeutic strategies, either as a primary therapy or as adjuvant radiation following surgery (10). The effects of radiation on patients are widely known (11-13); short-term effects mainly comprise damage to the skin and mucosa in the irradiated region, while long-term effects include xerostomia and an increased risk of secondary malignancies (14).

However, the effects of a previous irradiation on the tumor stroma are largely unknown. *In vitro*, it has been demonstrated that CAFs exhibit no significant changes in proliferation or growth when exposed to radiation (15), while another study indicated an enhanced capability of irradiated fibroblasts to promote survival of co-cultured cancer cells (16). In these previous studies, however, the irradiation was delivered *in vitro*, and the long-term effects on the irradiated tumor stroma were not investigated.

Our previous study demonstrated decreased viability of tumor cells and decreased interleukin (IL)-8 secretion when the tumor cells were co-cultured with fibroblasts from pre-irradiated human skin, as compared with skin-derived fibroblasts from non-irradiated patients (17). This raises the question of whether an irradiation of the head and neck during cancer therapy changes the properties of fibroblasts on a long-term basis, and what these changes consist of.

The primary objective of the present study was to evaluate the long-term effects of irradiation during therapy for HNSCC on skin-derived human fibroblasts compared with fibroblasts from non-irradiated skin, in terms of viability, apoptosis, necrosis, cell expansion and motility.

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Materials and methods

Acquisition and culture of fibroblasts. Fibroblasts were obtained from skin samples from 20 patients undergoing neck surgery at the University Hospital Würzburg, Germany, between October 2012 and November 2013. Of the 20 patients, 10 had been treated with intensity-modulated irradiation with 60-70 Gy for 6 weeks during head and neck cancer therapy 6-18 months previously (Table I). The other 10 patients underwent neck surgery for other reasons than cancer (Table I). Approval was obtained from the Ethics Committee of the Medical Faculty, University of Würzburg (approval no. 12/06), and informed consent was obtained from all patients involved. Tissue preparation was performed as described in our previous study (17), which included a modification of the protocol described by Vangipuram *et al* (18). In summary, the skin samples were cleared of fat and cut into small pieces of 2-3 mm, which were then seeded on 6-well plates. After 60 min of culture without medium at 37°C and 5% CO₂, the tissue pieces had sufficiently adhered to the bottom of the plates, such that Dulbecco's modified Eagle medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal calf serum (Biocrom, Ltd., Cambridge, UK), 100 U/ml penicillin and 100 µg/ml streptomycin [DMEM-expansion medium (DMEM-EM)] could be added without the pieces being washed away. From these tissue pieces, the fibroblasts grew out into the periphery. Every other day, the medium was replaced and passaging was performed when the cells had reached 70-80% confluence; passaging was performed by trypsinization (0.25% trypsin; Invitrogen; Thermo Fisher Scientific, Inc.), washing in PBS and seeding into new flasks or treatment wells.

Cell count. A total of 2×10^4 cells were incubated in DMEM-EM at 37°C with 5% CO₂ for 4 days, while electronically evaluating the cell number and cell viability each day using CASY® Technology (Innovatis AG, Reutlingen, Germany). Only cells labeled viable by the electronic counting were included in the analysis for the cell counting.

MTT assay. The MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) colorimetric staining method according to Mosmann (19) was used to study the viability of cells. All wells were incubated with 1 ml of MTT (1 mg/ml) for 5 h at 37°C with 5% CO₂. MTT was then removed and 1 ml of isopropanol was added, followed by another incubation period of 1 h at 37°C with 5% CO₂. Color changes due to the conversion of MTT to blue formazan dye were measured using a multi-plate reader (Titertek Multiskan PLUS MK II; Labsystems Diagnostics Oy, Helsinki, Finland) at a wavelength of 570 nm.

Annexin V/propidium iodide staining. A BD Pharmingen™ APC Annexin V kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to evaluate apoptosis. Cells in suspension and adherent cells were harvested and washed twice with PBS, followed by resuspension in 1:10 binding buffer (0.1 M HEPES, pH 7.4, 1.4 M NaCl, 25 mM CaCl₂) at a density of 1×10^6 cells/ml. Aliquots of this cell suspension (100 µl; 1×10^5 cells) were then transferred to a 5 ml culture tube. Propidium iodide (5 µl) and Annexin V-APC (5 µl) were added to each aliquot. Following

15 min of incubation at room temperature in the dark, the cells were resuspended with 400 µl 1:10 binding buffer. A FACS-canto flow cytometer (BD Biosciences) was used to analyze the samples. Propidium iodide staining indicated cells with damaged membranes.

Scratch assay. A scratch assay was used to analyze cell migration capability. Fibroblasts (1×10^5 cells/ml) were cultivated in a 12-well round-bottom plate at 37°C and 5% CO₂. After 24 h, a straight-line wound was induced with a sterile 1-ml pipette tip. Subsequently, the culture plates were washed with PBS and images were captured (day 0) with a Leica DMI 4000B Inverted Microscope at x40 magnification (Leica Microsystems GmbH, Wetzlar, Germany). The cells were then incubated for a further 24 h at 37°C with 5% CO₂, before images of the plates were captured (day 1) and the percentage of the wound closure was evaluated. This was repeated after another 24 h of incubation (day 2). The calculation of the area of the wound closure was investigated using ImageJ software (version 1.43u, open source product) at day 0, day 1 and day 2.

Statistical analysis. The data collected was transferred to standard spreadsheets and statistically analyzed using GraphPad Prism Software (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). The Gaussian distribution was tested via first column analysis. Students t-test followed by Tukey's multiple comparison test was used for statistical analysis. Data are presented as the mean ± standard deviation, unless otherwise stated. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cell count. In a consecutive cell count for 4 days, non-irradiated fibroblasts exhibited a constant increase in cell number between days 0 and 4, reaching a median of 1.15×10^5 cells on day 4. Fibroblasts from pre-irradiated tissue only had a minor increase in cell number, reaching a plateau on day 3 with a median of 4.96×10^4 cells (Fig. 1). The differences between the irradiated and non-irradiated groups were statistically significant on days 2, 3 and 4 ($P = 0.0002$, $P = 0.0001$ and $P = 0.0001$, respectively). At day 4, non-irradiated fibroblasts had a median cell viability of 76%, whereas pre-irradiated fibroblasts had a median viability of 66%.

MTT assay. Viability of non-irradiated and pre-irradiated fibroblasts was analyzed by MTT assay (Fig. 2). The assay revealed significantly lower cell viability for fibroblasts cultured from pre-irradiated skin tissue compared with non-irradiated fibroblasts ($P = 0.0061$).

Annexin V/propidium iodide analysis. The Annexin V/propidium iodide analysis was used to determine differences in the rates of apoptosis, necrosis and viability between pre-irradiated fibroblasts and non-irradiated fibroblasts (example shown in Fig. 3). Significantly higher rates of apoptosis ($P = 0.0080$) and necrosis ($P = 0.0019$) were observed in pre-irradiated fibroblasts compared with non-irradiated fibroblasts (Fig. 4). A lower percentage of viable cells in pre-irradiated fibroblasts ($P = 0.0002$; Fig. 4) was also observed compared with

Table I. Data and characteristics of the patients.

Patient no.	Sex	Age, years	Primary tumor site/ reason for surgery	Radiation dose, Gy	Concurrent chemotherapy
1	Male	63	Hypopharynx	70	Yes
2	Male	54	Oropharynx	66	No
3	Male	59	Larynx	60	No
4	Female	72	Hypopharynx	69	Yes
5	Male	57	Larynx	60	No
6	Male	61	Hypopharynx	69	Yes
7	Female	61	Oropharynx	69	Yes
8	Female	70	Larynx	60	No
9	Male	59	Oropharynx	69	Yes
10	Male	65	Hypopharynx	66	No
11	Female	46	Parotidectomy	n/a	n/a
12	Female	56	Cervical cyst	n/a	n/a
13	Male	39	Cervical cyst	n/a	n/a
14	Female	65	Parotidectomy	n/a	n/a
15	Male	72	Cervical cyst	n/a	n/a
16	Male	81	Cervical cyst	n/a	n/a
17	Male	59	Parotidectomy	n/a	n/a
18	Female	66	Cervical cyst	n/a	n/a
19	Male	69	Parotidectomy	n/a	n/a
20	Male	62	Parotidectomy	n/a	n/a

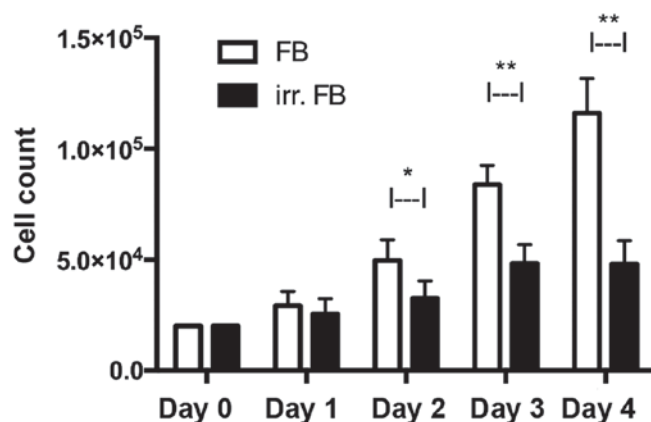


Figure 1. Consecutive electronic cell count for 4 days. There was a constant increase in cell numbers in the non-irradiated fibroblasts and only a minor increase in pre-irradiated fibroblasts. The higher cell numbers in non-irradiated vs. irradiated fibroblasts were statistically significant on days 2, 3 and 4 (*P=0.0002 and **P=0.0001). FB, fibroblast; irr., irradiated.

non-irradiated fibroblasts, thus confirming the results of the MTT-assay.

Scratch assay. The scratch assay was used to evaluate cell migration into a wound area in monolayer conditions. When creating the wound on day 0, no statistically significant difference was observed between the two groups. Following periods of 24 and 48 h, respectively, at 37°C and 5% CO₂, the wound closure was measured and compared between the two groups. The pre-irradiated fibroblasts showed significantly slower wound closure compared with non-irradiated fibroblasts on

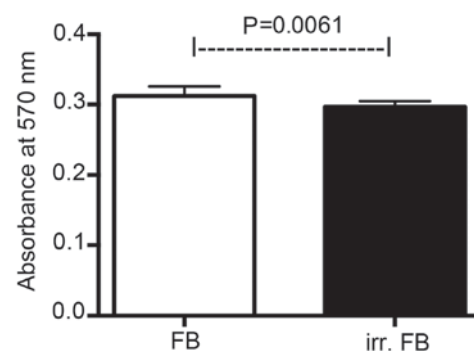


Figure 2. MTT assay. There was a statistically significant decrease in cell viability in pre-irradiated fibroblasts compared with non-irradiated fibroblasts (P=0.0061). FB, fibroblast; irr., irradiated.

day 1 and day 2 (P=0.0001 and P=0.0027, respectively), thus indicating reduced cell motility (Figs. 5 and 6).

Discussion

The present study focused on the effects of a previous radiation on the properties of skin-derived fibroblasts. Tumor progression has been recognized as the product of an evolving crosstalk between different cell types within the tumor and its surrounding supporting tissue, or tumor stroma (20). The immune cells, capillaries, basement membrane, activated fibroblasts and extracellular matrix surrounding the cancer cells constitute the tumor stroma (21). Fibroblasts comprise a major component of the tumor stroma, and numerous studies have indicated a prominent role for these cells in

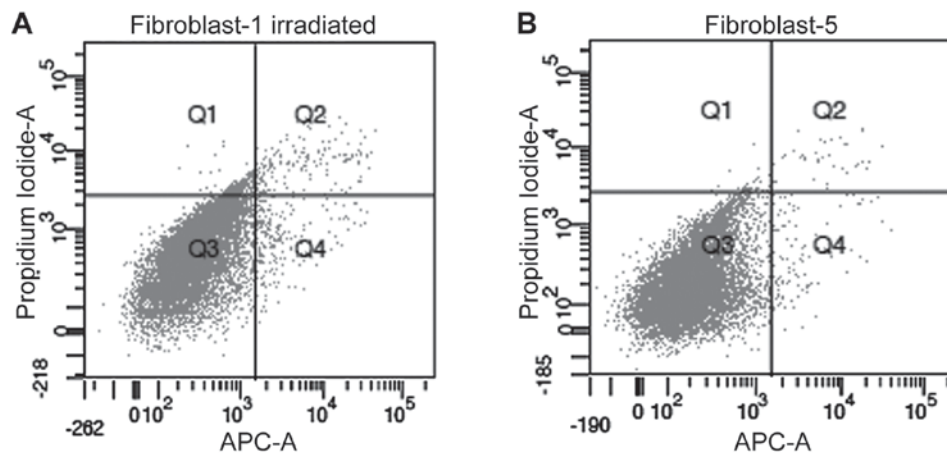


Figure 3. Annexin V-propidium iodide assay. Representative results from (A) non-irradiated fibroblasts and (B) pre-irradiated fibroblasts are shown. Q1, % of damaged cells; Q2, % of necrotic cells; Q3, % of viable cells; Q4, % of apoptotic cells. APC-A, allophycocyanin-A.

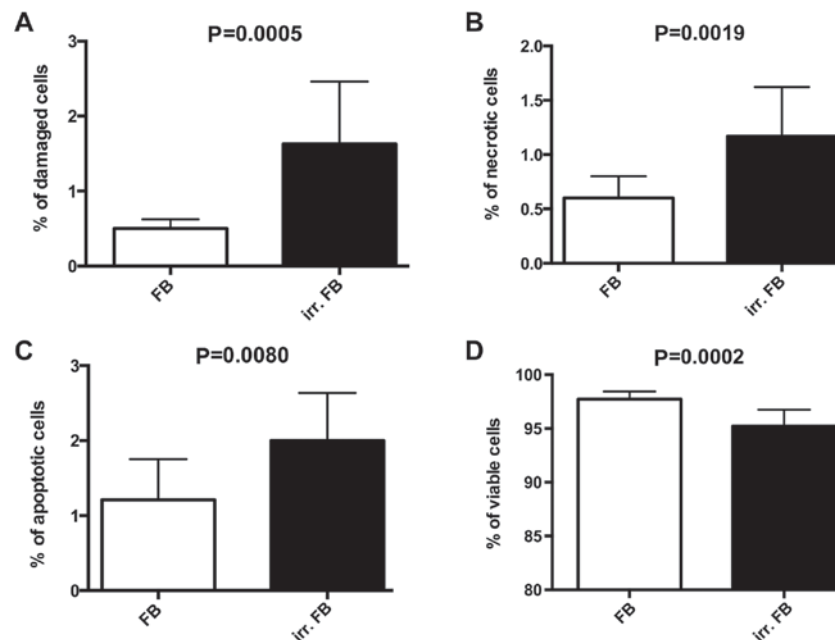


Figure 4. Statistical evaluation of the Annexin V-propidium iodide assay. There were increased rates of (A) damaged cells ($P=0.0005$), (B) necrotic cells ($P=0.0019$), and (C) apoptotic cells ($P=0.0080$), as well as (D) a lower percentage of viable cells ($P=0.0002$) in pre-irradiated fibroblasts compared with non-irradiated fibroblasts. FB, fibroblast; irr., irradiated.

cancer progression and metastasis (2,22). CAFs have been established as key components of tumor progression, and increasing information indicates that they possibly contribute to a wide range of fibrotic stromal programs of numerous different tumors (23,24). In the context of a highly dynamic and injurious tissue microenvironment, including damage induced by chemotherapy or radiotherapy, CAFs may represent a resistant stromal cell type that may be involved in tumor relapse (25).

Particularly in regard to relapsing cancer, information about whether previous radiation changes the properties and behavior of fibroblasts is desirable. It is already known that cells exposed to radiation may survive, but give rise to progeny that carry heritable damage (26). This damage may become lethal during many generations of division cycles of the originally irradiated progenitor cell (27,28). Gorgojo and Little (29)

previously described the expression of lethal mutations in the progeny of irradiated mammalian cells, thus showing an effect on surviving cells a long time after the irradiation was administered. Chang and Little (27,28) reported delayed reproductive deaths in cell clones surviving irradiation, several generations following therapy. However, these experiments were performed on established cell lines grown and irradiated *in vitro*, and the relevance of lethal mutations to irradiation of cells *in vivo* has been uncertain (30). Chatterjee *et al* (30) stated that the reduction in the long-term viability of irradiated cell populations appears to be dose-dependent and is most noticeable following large doses of radiation. In the present study, fibroblasts derived from pre-irradiated skin showed significantly lower viability and slower cell growth compared with skin-derived fibroblasts from non-irradiated patients, thereby confirming the *in vitro* data available in the literature.

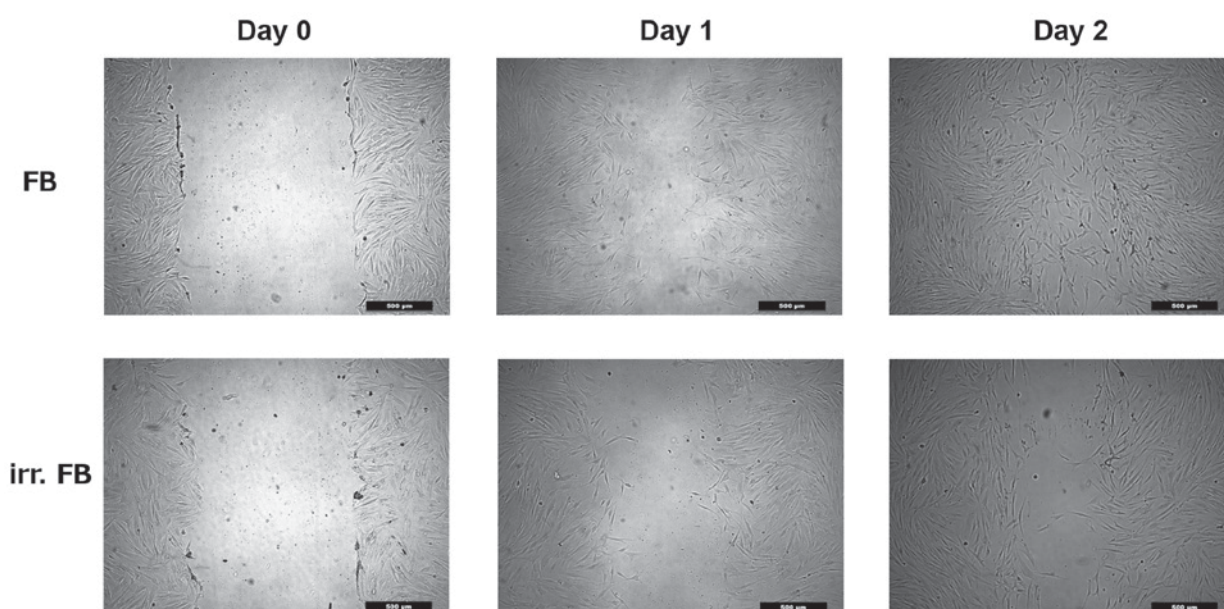


Figure 5. Representative images of the scratch assay. Upper row, non-irradiated fibroblasts. Lower row, pre-irradiated fibroblasts. On day 0, the wound area was the same in the two groups (left). On day 1, a lesser degree of wound closure was observed in pre-irradiated fibroblasts than non-irradiated fibroblasts (middle). On day 2, the wound area in non-irradiated fibroblasts was almost completely closed, while the cell-free area was still clearly visible in the pre-irradiated fibroblasts (right). Scale bar, 500 μm . FB, fibroblast; irr., irradiated.

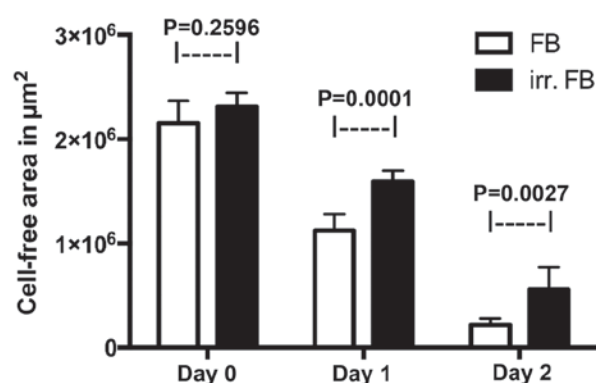


Figure 6. Statistical analysis of the scratch assay. There was a significantly lower percentage of wound closure on days 1 and 2 in the pre-irradiated fibroblasts compared with non-irradiated fibroblasts. FB, fibroblast; irr., irradiated.

Contrary to the aforementioned studies, the cells used in the present study were primary human fibroblasts from skin irradiated 6-18 months before, thus more accurately representing the real physiological effects of irradiation *in vivo*.

The levels of apoptosis and necrosis were elevated in the pre-irradiated fibroblasts in the present study. This indicated that there is more than one mechanism by which irradiation damages surviving cells. O'Reilly *et al* (31) reported a constant frequency of non-lethal mutations occurring per cell division, indicating a permanent genetic change induced by radiation. Kadhim *et al* (32) also favored this hypothesis, speculating that this mechanism may lead to cell death by an active process such as apoptosis, rather than necrosis. O'Reilly *et al* (31) also demonstrated abnormalities in irradiated cultures a number of generations after initial exposure, including convolution of the nuclear envelope, increased incidence of microvilli and lysosomal accumulations, which are characteristic of apoptosis

rather than necrosis. However, early senescence as an alternative cause of radiation-induced changes has been discussed for mesenchymal stem cells (MSCs) and fibroblasts (33,34).

In the present study, the scratch assay revealed reduced motility of pre-irradiated fibroblasts. Rodriguez-Menocal *et al* (35) demonstrated decreased motility and migration capability of MSCs in an irradiated murine delayed wound healing model. Henke *et al* (36) reported decreased motility and contractility in prostate CAFs, in their study associated with an increase of focal adhesion kinase. By contrast, Nicolay *et al* (37) found no changes in the actin cytoskeleton or in the functional motility of irradiated MSCs and fibroblasts. However, whether the delayed wound healing observed in the present study is the result of reduced motility due to radiation-induced genetic changes or a consequence of the reduced cell division remains unclear.

The effects of the radiation-induced changes observed in the fibroblasts in the present study of tumor cells differed from the data available in previous studies. Kamochi *et al* (38) presented data indicating that irradiated fibroblasts promote growth and invasion of co-cultured HNSCC. Other studies also reported of increased invasiveness of pancreatic and mammalian tumor cells co-cultured with irradiated fibroblasts (39,40). However, in all these studies the radiation was administered *in vitro* to the fibroblasts, so the long-term effects could not be examined. The use of fibroblasts from human skin, which has been exposed to therapeutic irradiation a number of months prior, appears to be more comparable to the physiological conditions *in vivo*. Using this approach, previous studies have already demonstrated a decrease in viability of HNSCC co-cultured with pre-irradiated fibroblasts (17). In addition, fibroblasts from pre-irradiated human skin decreased the secretion of IL-8 by HNSCC cells in a co-culture of these two cell types (17).

A notable drawback of the present study was that functional analysis regarding cytokine secretion and protein

synthesis was not included. A quantitative evaluation of the secretory profile of the fibroblasts with or without radiation may elucidate the mechanisms behind the changes observed in the present study. In particular, ILs such as IL-6 and IL-8 have been shown to be prominent modifiers of cancer cell behavior (41-44). Whether the amount of these ILs produced by fibroblasts changes following irradiation, however, has not been investigated thus far. These analyses will be part of future studies at our institution.

In conclusion, previous irradiation is associated with changes in the properties of fibroblasts derived from human skin in the irradiated area. Reduced cell viability, increased rates of apoptosis and necrosis, slower cell growth and reduced cell motility may be demonstrated. Since the effects of these radiation-induced changes of the fibroblasts on tumor cells have already been demonstrated, more information regarding the genetic and secretory alterations of the fibroblasts are warranted to fully elucidate the long-term effects of radiation. These radiation-induced changes in fibroblasts (and, therefore, the tumor stroma) may be a possible novel target for therapeutic strategies for recurring cancer, and therefore require additional investigation.

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