In Vitro Radiosensitivity of Fibroblasts from Thyroid and Skin Cancer Patients Treated with X-Rays for Tinea Capitis[1]

Elaine Ron,[2] Robert E. Tarone, Baruch Modan, Rina Chaki, Esther Alfandary, Dilys M. Parry, Michael Makar, Neva Setlow, John J. Mulvihill, Robert W. Miller, and Richard B. Setlow

Epidemiology and Biostatistics Program, National Cancer Institute, NIH, Bethesda, MD 20892 [E. R., R. E. T., D. M. P., R. W. M.]; Departments of Clinical Epidemiology and Genetics, Chaim Sheba Medical Center, Tel Hashomer, Israel [B. M., R. C., E. A.]; Medical and Biology Departments, Brookhaven National Laboratory, Upton, NY 11973 [M. M., N. S., R. B. S.]; and Department of Human Genetics, University of Pittsburgh, Pittsburgh, PA 15260-0001 [J. J. M.]

Abstract

To investigate the hypothesis that persons who developed thyroid or skin cancer subsequent to scalp irradiation for tinea capitis are particularly sensitive to radiation, possibly because of a high frequency of ataxia-telangiectasia, we used an *in vitro* cell survival assay to evaluate radiosensitivity of their fibroblast cell strains. Study subjects were selected from a cohort of 10,834 Israelis irradiated during childhood for tinea capitis. Skin fibroblasts were obtained from thyroid and skin cancer patients (cases) as well as a sample of subjects who did not have cancer (controls). Fibroblasts were cultured and then loss of colony-forming ability as a result of acute X-irradiation was evaluated. Comparison of survival curve parameters (mean inverse of the slope and the dose needed to reduce colony survival to 10%) between 12 thyroid cancer and 12 control strains showed no differences ($P > 0.5$). A slightly increased radiation sensitivity of the skin cancer cases compared with their controls was observed. Although based on few subjects (14 cases and 11 controls), the findings were similar whether the mean inverse of the slope ($P = 0.06$) or the dose needed to reduce colony survival to 10% ($P = 0.05$) was evaluated. However, because of the small size of the study and potential errors inherent in survival assays, our finding that cell strains derived from patients who developed skin cancer exhibit enhanced radiosensitivity should be viewed as preliminary and interpreted cautiously.

Introduction

A cohort of 10,834 Israelis who received scalp irradiation during childhood as treatment for tinea capitis (scalp ringworm) exhibited excess risks of benign and malignant head and neck tumors, compared with 16,226 nonexposed subjects (1–3). The risk of nonmelanoma skin cancer of the head and neck was significantly elevated (relative risk = 4.2) at doses of about 700 cGy (2). A 4-fold risk of thyroid cancer also was demonstrated even though the thyroid gland was exposed to scatter radiation only, resulting in an average dose of 9 cGy (3). Because the excess relative risks/cGy were large compared with other studies of childhood irradiation (4, 5), we looked for evidence of an interaction between host susceptibility and radiation exposure.

The tinea capitis cohort is largely (60%) comprised of Jews who immigrated from North Africa where the frequency of AT[3] is unusually high (6, 7). Because AT patients, and possibly AT heterozygotes (e.g., parents of AT patients), are at increased risk of cancer and exhibit marked acute radiosensitivity clinically, and their cells are abnormally sensitive to *in vitro* ionizing radiation, we considered the possibility that some of the radiation effect observed was a result of a high frequency of AT heterozygotes among the cancer cases (8–13). The frequency of AT is between 1/40,000 and 1/100,000 in the general population but based on the Hardy-Weinberg equilibrium principle approximately 1% of the population would be AT heterozygotes (8, 9). Levin and Perlov (6) estimated the frequency of AT in Jews who immigrated to Israel from Morocco and Tunisia to be about 1/8000. On the basis of the Hardy-Weinberg principle, AT heterozygotes would comprise about 2% of this North African Jewish population.

To investigate the hypothesis that members of the tinea capitis cohort who developed cancer are particularly sensitive to radiation, possibly because of a high frequency of AT heterozygotes, we used an *in vitro* cell survival assay to evaluate radiosensitivity of fibroblast cell strains derived from skin biopsies obtained from irradiated study subjects. Thyroid and skin cancers were selected for study because of the high excess relative risk associated with these cancers in the tinea capitis cohort.

Methods

Study Subjects and Skin Biopsies. Study participants were selected from the Israel tinea capitis cohort of 10,834 irradiated subjects. The methods employed in this study have been described previously (1–3). Patients who developed

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[2] To whom requests for reprints should be addressed, at National Cancer Institute, NIH, Division of Cancer Etiology, 6130 Executive Blvd., EPN 408, Rockville, MD 20852.

[3] The abbreviations used are: AT, ataxia-telangiectasia; MEM, modified Eagle's medium; D_0 inverse of the slope; D_{10} the dose needed to reduce colony survival to 10%.

thyroid or skin cancer comprised the case group. A sample of irradiated subjects who did not have cancer were selected as controls. The controls were matched to the thyroid and skin cancer cases by age, sex, and number of radiation treatments. After receiving informed consent from each study subject, skin biopsies were taken from 17 thyroid cancer patients, 16 thyroid cancer controls, 36 skin cancer patients, and 29 skin cancer controls. Biopsies were taken by a specially trained registered nurse in hospital outpatient clinics or in the patient's home. The skin in the inner forearm was prepared antiseptically and then scraped, with minimal discomfort, using a 4-mm razor. A specimen of approximately 5 mm in length x 1–2 mm in depth was taken. Each biopsy was placed in sterile tissue culture medium (MEM) in a plastic sterile collection bottle and transferred immediately in a well insulated transport case to the tissue culture facility of the Cytogenetics Department of the Chaim Sheba Medical Center.

Method of Culturing Fibroblasts. After arriving in the laboratory, the skin tissue was washed thoroughly and transferred to a 60-mm covered petri dish (Falcon, Oxnard, CA) containing a culture medium of 70% Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) and 30% fetal bovine serum (GIBCO). Mycostatin, penicillin, and streptomycin were added and the medium was tested for mycoplasma. The tissue was cut into 1- to 2-mm pieces and placed in 25-cm^2 tissue culture flasks (Falcon), which were maintained at 37°C in a humidified incubator with 10% CO_2. Half of the medium was changed twice a week for about 4 weeks. The fibroblasts that grew out were suspended with trypsin-versene solution (Bio-Lab, Jerusalem, Israel). With the help of an inverted microscope, the proportion of trypsinized cells was determined. When most cells trypsinized, cells were transferred to another 25-cm^2 flask. Clumps of cells were separated, more culture medium was added, and again cells were incubated. When cells became confluent they were transferred to 75-cm^2 flasks. Cells were reseeded into 75-cm^2 flasks once more. Eighty-one (83%) cultures were grown successfully.

After the cultures were established, they were subdivided and coded. From each sample, two flasks of fresh cell lines were frozen. In batches, four other flasks/specimen were shipped to the fibroblast repository under contract to the National Cancer Institute in the United States. Soon after, fresh cell lines were transferred to Brookhaven National Laboratory where the survival assays were performed.

X-Ray Survival Assay. Radiation-induced loss of colony-forming ability as a result of acute X-irradiation was evaluated using two assay methods. In both methods cells were grown in flasks until near confluence. In method G, cultures of cells at this stage were split and passaged once, and then grown again to confluence in 25-cm^2 dishes. In this method, the cells were washed two times with phosphate-buffered saline and trypsinized. The cells were resuspended in 10 ml α-MEM at about 10^6 cells/ml. Cells were diluted to 5 x 10^4 ml and 2 ml of this suspension were distributed into 15-ml centrifuge tubes. The tubes plus the suspended cells were irradiated at room temperature with 250-kVp X-rays at 30 mA, with 0.5 mm Cu and 1.0 mm Al external filtration at a rate of 1.0 Gy/min, and received doses of 0, 0.5, 1.0, 2.0, 3.0, 4.0, and 6.0 Gy. Then the cells were diluted in α-MEM and plated (5 plates/dose point). Preliminary experiments indicated that survival determined using method G was indistinguishable from survival determined after irradiation of exponentially growing cells.

Method F was generally similar to method G but it allowed time for recovery from potentially lethal damage. The cells were irradiated in flasks while confluent and permitted to incubate for 24 h before trypsinizing, diluting, and plating as described above. One would expect that this method might reveal results somewhat similar to those for chronic irradiation of confluent cultures because both methods leave time for repair before plating.

For all but three cases, two or more experiments were performed. A total of 56 cell strains were evaluated in three series: 12 thyroid cancer cases and 12 controls were studied between August 1987 and May 1989; 11 skin cancer cases and 10 controls were studied between December 1988 and September 1989 (called skin series I); and 3 skin cancer cases and 8 controls were studied between October 1989 and January 1990 (called skin series II). Some additional experiments were repeated later. Colonies with 50 or more cells were scored, and plating efficiencies and survival fractions were calculated. All radiation survival experiments were done without knowledge of whether the strain came from a cancer or noncancer subject.

Statistical Analysis. Survival capability was defined as the ratio of the plating efficiency after X-ray exposure relative to the unirradiated base-line measurement in the same experiment. Two methods of analysis were used for estimating and comparing the survival curves. In the first, as in Ban *et al.* (14), the data were fit to a multitarget survival expression that gave the parameters of interest. In the second, described in detail by Tarone *et al.* (15), the intercept and the slope for each survival curve were estimated by least squares linear regression in the exponential portion of the dose-response curve. Because the two methods gave very similar results and conclusions, we present only the findings of the latter method (15). Multiple experiments were performed on the same cell strain, and summary estimators of the survival parameters D_0 and D_{10} were derived from the survival curves using the partially weighted mean or a semiweighted mean method depending on the degree or heterogeneity of the individual parameter estimates (15). The partially weighted mean was used when the slope or D_{10} estimates were homogeneous; the semiweighted mean was used when they were heterogeneous. The semiweighted mean method gives less weight to experiments with a high error variance. The D_0 value summarizes the survival curve in the high dose range i.e., the exponential portion of the survival curve. The D_{10} value reflects both the width of the initial shoulder and the exponential portion of the curve, and therefore may be a better total measure of the response of a strain to radiation. Analysis of variance methods were used to combine the results from the two skin cancer series.

Because the G and F assay methods are independent, we also were able to combine the results from the two assays using the same statistical methods. A two-tailed *t* test was used to compare the radiation sensitivity of the cell strains from persons with cancer with the control cell strains. Log transformations of all survival parameters were performed before making comparisons between cases and controls. Even though the controls originally were matched individually to the cases, the statistical analysis is based on a nonmatched population because of patient refusals, growth failure, and early termination of the study.

Results

The cases and controls in the two series were alike in terms of gender, mean year of birth and irradiation and number

Table 1 Summary of selected characteristics of the study population

Characteristic	Thyroid cancer		Skin cancer	
	Cases	Controls	Cases	Controls
Total subjects	12	12	14	18
Females	10 (83.3%)	8 (66.7%)	9 (64.3%)	12 (66.7%)
Born in North Africa	8 (66.7%)	2 (16.7%)	3 (21.4%)	5 (27.8%)
Mean year of birth	1947.2	1948.5	1948.2	1946.8
Mean age at irradiation	6.5	7.2	6.8	7.4
Mean organ dose (cGy)	10.0	8.2	807.4	651.7
Mean experiments/strain	3.5	2.9	2.3	2.2

of experiments/strain (Table 1). However, consistent with results from our earlier study (2), more thyroid cancer cases were born in North Africa than their controls or the skin cancer cases or controls. The mean radiotherapy dose was comparable for the thyroid cancer cases and controls but was higher for the skin cancer cases than their controls.

The X-ray survival parameters for the thyroid cancer series are presented in Table 2. With method G, D_0 values ranged from 0.91 to 1.30 Gy and D_{10} values ranged from 2.11 to 2.96 Gy. Comparison between the thyroid cancer and control strains' mean D_0 and D_{10} values showed no differences ($P > 0.5$).

When method F was used, the values of the survival parameters were close to 60% larger than the values obtained using method G. This difference in the values of the survival parameters reflects the ability to repair sublethal cellular damage. However, because the correlation between the two methods was excellent ($P < 0.001$), method F provided essentially the same results as method G in terms of the comparison in radiosensitivity between the case and control strains.

Survival parameters for the skin cancer and control strains (Table 3) were analyzed initially as two individual series because the experiments were conducted in two batches with a 2-month hiatus between them. The mean values of the survival parameters for skin series I were considerably higher than either the thyroid series or the later skin series (series II). Attempts to determine why this was so were unproductive. The same technician working with the same senior scientist carried out the experiments for all three series. In general, the cells in series I were very good growers and the time between plating and achievement of confluence was on average 2–3 days shorter than in the other series. The slightly different growth pattern may have affected the absolute values of the survival parameters.

In the first skin cancer series, the method G D_0 and D_{10} values for the strains derived from the cancer patients ranged from 1.06 to 1.30 Gy and 2.62 to 3.55 Gy, respectively. For strains derived from the controls, the range of D_0 values was from 1.10 to 1.32 Gy and the range of D_{10} values from 2.82 to 3.71 Gy. The mean D_0 value was lower for the skin cancer strains and this difference was of borderline significance ($P = 0.06$). In the second skin cancer series, the number of cases was considerably smaller; however, as seen in the table the case strains had lower mean values for both survival parameters. When the two series were combined, the greater radiosensitivity of the case strains reached ($P = 0.03$) or nearly reached ($P = 0.06$) statistical significance, as reflected by D_{10} and D_0 values, respectively.

The survival parameters using method F were again much higher than when using method G. On the basis of the series I skin data only, the findings were in the same direction as those seen using method G. The cases had lower mean D_0 and D_{10} values than the controls, but the difference between the study groups was not statistically significant (Table 3). When the results from methods G and F were pooled, the difference between cases and controls became stronger ($P = 0.06$ and $P = 0.05$ for D_0 and D_{10}, respectively).

Table 2 Survival curve parameters using methods G and F for thyroid cancer cases and control fibroblast strains

Study group	n[a]	D_0 (cGy)		D_{10} (cGy)	
		Mean	SE	Mean	SE
Method G					
Cases	12	105.8	2.4	261.9	8.2
Controls	12	107.0	3.2	257.8	6.2
		$P = 0.8$		$P = 0.7$	
Method F					
Cases	12	166.3	4.8	396.1	9.9
Controls	12	168.5	5.4	400.0	14.9
		$P = 0.8$		$P = 0.8$	

[a] n, number of patients.

Table 3 Survival curve parameters using methods G and F for skin cancer cases and control fibroblast strains

Study group	n[a]	D_0 (cGY)		D_{10} (cGy)	
		Mean	SE	Mean	SE
Skin series I: method G					
Cases	11	114.5	2.3	293.0	8.0
Controls	10	121.1	2.3	310.5	8.2
		$P = 0.06$		$P = 0.13$	
Skin series II: method G					
Cases	3	100.0	3.6	241.0	16.0
Controls	8	105.8	3.8	266.9	8.7
		$P = 0.45$		$P = 0.17$	
Series I and II: method G		$P = 0.06$		$P = 0.03$	
Skin series I: method F					
Cases	11	218.4	10.8	514.2	19.0
Controls	10	230.2	9.0	534.6	22.4
		$P = 0.35$		$P = 0.49$	
Methods G and F		$P = 0.06$		$P = 0.05$	

[a] n, number of patients.

Discussion

Results of *in vitro* cell survival experiments suggest that Israeli patients developing thyroid cancer after scalp irradiation as treatment for tinea capitis are not hypersensitive to

radiation as measured by colony-forming ability. The slightly increased radiation sensitivity of the skin cancer cases compared with their controls was notable. Although based on few subjects, the findings were similar whether the mean D_0 or D_{10} was evaluated. In addition, results were consistent, although the magnitude differed, regardless of which of the two survival assays was used.

In a study of atomic bomb survivors, there was no evidence that women who developed breast cancer were more sensitive to the damaging effects of radiation than women who did not develop breast cancer (14). In that study, 54 cell strains from exposed and nonexposed breast cancer cases and controls were compared and no difference in radiosensitivity was reported between exposed and nonexposed groups, nor between breast and non-breast cancer patients. However, only 12 of the 30 exposed patients had received radiation doses of >=0.5 Gy and women exposed to less than 0.5 Gy have not exhibited a particularly high excess risk of breast cancer.

Although our generally negative findings are consistent with those of the atomic bomb survivors, they were of interest because it was hypothesized that our study population might have an elevated susceptibility to radiation damage because of an expected high frequency of AT heterozygotes. However, detecting small differences in radiation response among individuals is difficult at the present time and our results may reflect the limitations in the assays employed. Colony forming assays can discriminate AT homozygotes well (12). However, even though AT heterozygotes as a group display enhanced radiosensitivity (16–18), this method has not been able to identify individual AT heterozygotes to date because of the considerable overlap of survival parameters between AT heterozygotes and normal donors (18).

Cell survival assays have several sources of potential error which would diminish the ability to detect host variation in radiosensitivity: error variance associated with observed measurements within individual experiments; interexperimental variability; inherent differences within groups of cell lines in which the range of normal values can be rather large; and systematic changes in laboratory procedures. In this study, the variation in the magnitude of the survival parameter values over time suggests that a change in the method of cell growth, storage conditions, or in the survival assay itself occurred. Survival assays also are subject to other types of variation. For example, there has been a report that skin fibroblasts derived from different body sites may differ in radiosensitivity, with skin fibroblasts being more sensitive than lung fibroblasts (19). In addition, there may be differences in measurement variability depending on the type of sample used; e.g., T-lymphocytes *versus* skin fibroblasts (20). It is not clear how much error variation may have affected our findings.

Thus, although no significant difference in radiosensitivity between irradiated patients with and without thyroid cancer was observed our findings do not negate the possibility that there are differences which were not discernable by the methods used. Our finding that patients developing skin cancer may have enhanced radiosensitivity is interesting, but because of the limitations described above, the results should be interpreted cautiously. Further studies should await an assay which is more powerful for detecting AT heterozygotes.

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