Advances in Brief

Response of Dermal Fibroblast Cultures from Patients with Unusually Severe Responses to Radiotherapy and from Ataxia Telangiectasia Heterozygotes to Fractionated Radiation[1]

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Abstract

We examined the cytotoxic effects of radiation delivered in daily fractions at clinically relevant doses in plateau phase cultures of skin fibroblast cell strains derived from ataxia telangiectasia (AT) heterozygotes, patients with unusually sensitive responses to radiotherapy, apparently normal patients, and cell bank controls. A gradual linear reduction in surviving fraction *versus* total dose was observed in the control group, comprised of apparently normal individuals and one patient with a normal clinical response to radiotherapy, after exposure to daily fractions of 2.0 Gy. There was a much steeper decline in surviving fraction among the AT heterozygotes and the group with sensitive responses to radiotherapy, such that after six daily fractions of 2.0 Gy (12 Gy total dose), the mean surviving fraction of the control group was significantly different from that of the AT heterozygotes ($P = 0.0009$) and that of the patients with unusually sensitive responses to radiotherapy ($P = 0.0002$). We propose that this assay may be a useful means of identifying cell strains from AT heterozygotes. Based on these results, the hypothesis is discussed that patients who suffer unusually sensitive clinical reactions to radiotherapy may be AT heterozygotes.

Introduction

Fractionated radiotherapy plays an integral role in the management of human cancer. However, local control rates and cure are limited by tumor radioresistance and normal tissue tolerance (1). Variability in normal tissue radioresponse has been reported in both clinical and laboratory studies (2–6). Hypersensitivity to ionizing radiation is an established hallmark of the autosomal recessive syndrome AT[3], but has also been reported in a few cases where the patients were without obvious genetic disorders (7, 8).

In earlier studies, radiation survival parameters were measured in dermal fibroblasts derived from patients who exhibited severe acute or late effects of radiation therapy to determine whether the radiation response *in vitro* correlated with clinical normal tissue reactions (9–11). Fibroblasts derived from such patients did not demonstrate the hypersensitive radiation response seen in AT homozygote fibroblasts; however, survival parameters of cells from patients with severe clinical responses were in the lower range seen in fibroblasts from apparently normal cell bank controls, and similar to the survival parameters of fibroblasts from AT obligate heterozygotes (12–14).

In the present investigation, we examined the effects of irradiation at clinically relevant daily doses similar to fractionated radiotherapy in plateau phase cultures of dermal fibroblasts derived from patients exhibiting severe clinical responses to radiotherapy, AT obligate heterozygotes, and normal cell bank controls. We chose this fractionation scheme in order to magnify the slight differences seen in radiation response within these cell strains and to take into account possible differences in the kinetics of recovery and DNA repair which may not be manifested in single dose X-ray survival curves.

Materials and Methods

The source and relevant patient information for each fibroblast strain used in this study are listed in Table 1. All cells were studied between passages 6 and 10 (12–20 mean population doublings). The cells were grown in Eagle's MEM (GIBCO) supplemented with 10–20% fetal bovine serum (Sigma), penicillin (50 units/ml), and streptomycin (50 μg/ml). The cells were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere.

Cells used for experiments were grown to plateau phase in 60-mm dishes (Costar). The culture medium was then changed daily for 3 days. At this time, the percentage of cells in S-phase as determined by pulse labeling with [^{3}H]thymidine and autoradiography was less than 2%. For the determination of graded single-dose survival curves (0–6.0 Gy), cultures were irradiated 24 h after the last medium change, subcultured immediately after irradiation, and seeded at low density to determine cell survival. For fractionation experiments, replicate cultures were irradiated with 2.0 Gy daily beginning 24 h after the last medium change. After irradiation, one dish was immediately subcultured for cell survival, and the remainder was returned to the incubator. This fractionation protocol was continued for up to 8 days with 24-h intervals between irradiations. Replicate, nonirradiated cultures of each cell strain grown and maintained in the same manner were subcultured with each daily fraction to measure cloning efficiencies. The culture medium was not renewed during the course of the fractionation experiments. The cloning efficiencies of the nonirradiated cultures remained constant. To determine cell survival, cells were subcultured and

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[3] The abbreviation used is: AT, ataxia telangiectasia.

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Table 1 Patient information and source of cell strains used in this study

	Age[a]	Sex	Source
Control group			
AG01519	3 day	M	NIA/CIMR[b]
AG01522	3 day	M	NIA/CIMR
RMP-2	26	F	HSPH
RMP-3	18	F	HSPH
JL-10	68	F	HSPH
Patients with sensitive responses			
JL-7	40	F	HSPH
JL-9	66	F	HSPH
PO-1	78	F	HSPH
GB-1	11	M	HSPH
AT heterozygotes			
GM03396	42	F	NIGMS/CIMR
AG03057	46	F	NIA/CIMR
AG03059	47	M	NIA/CIMR
AT homozygotes			
GM02052	15	F	NIGMS/CIMR
AG03058	14	F	NIA/CIMR

[a] Patient age at time of biopsy.

[b] NIA/CIMR, Aging Cell Repository, Coriell Institute for Medical Research; HSPH, Laboratory of Radiobiology, Harvard School of Public Health; NIGMS/CIMR, Genetic Mutant Cell Repository, Coriell Institute for Medical Research.

seeded into 100-mm dishes at two cell densities per dose designed to yield approximately 50–100 viable colonies. The dishes were returned to the incubator for 14 to 21 days when macroscopic colonies were stained and counted.

Irradiations were performed with either a Philips MCN 165 industrial X-ray machine operated at 100 kVp and 15 mA (inherent filtration 1 mm beryllium, with 3 mm aluminum added filtration), delivering a dose rate to the cells of 0.52 Gy/min, or a U.S. Nuclear GR-9 cobalt 60 source delivering γ rays at 6.0–8.0 Gy/min. Similar results were obtained with either irradiator.

The data from two to four separate experiments were pooled to obtain the survival data presented for each cell strain. The survival parameters D_0, D_{10}, and N were obtained by linear regression analysis of the single-dose survival curve data. The mean inactivation dose ($\bar{D}$) was derived by use of a linear quadratic model with α and β values (15). The SF_2 and SF_{12} values were taken directly from the 2.0 Gy or 12 Gy survival data.

Results

The survival curves of human fibroblast cell strains irradiated in plateau phase and immediately subcultured and replated to low density are shown in Fig. 1. The radiobiological survival parameters for the fibroblast strains used in this study are shown in Table 2. These values were determined by linear regression analysis of the data from single-dose, graded survival curves of cells irradiated in the plateau phase and subcultured immediately thereafter. The control group is comprised of two apparently normal strains derived from foreskins (AG01519 and AG01522), two apparently normal strains from women who underwent reduction mammoplasty (RMP-2 and RMP-3), and a breast cancer patient who received radiotherapy without experiencing severe skin reactions (JL-10). The SF_2 values in this group ranged from 0.120 to 0.306 (mean, 0.217). The D_0 values (inverse of the slope) ranged from 0.93 to 1.26 Gy, and the D_{10} values (dose required to reduce the surviving fraction to 10%) ranged from 2.17 to 3.41 Gy. The $\bar{D}$ values ranged from 0.94 to 1.60 Gy.

In the group of patients who had severe reactions to radiotherapy, the SF_2 values ranged from 0.093 to 0.148 (mean, 0.125), the D_0 values ranged from 0.84 to 0.96 Gy, and the D_{10} values ranged from 1.93 to 2.35 Gy. The $\bar{D}$ values ranged from 0.82 to 1.03 Gy. This group is comprised of two patients with breast carcinoma who had severe acute reactions to radiotherapy (JL-7 and JL-9), one patient with Kaposi's sarcoma (non-AIDS related) with an acute reaction to radiotherapy (PO-1), and a patient irradiated as an infant for histiocytosis X who suffered a severe late reaction many years later (GB-1).

In the three strains from obligate heterozygote patients (parents of AT homozygotes), the SF_2 values ranged from 0.101 to 0.132 (mean, 0.121), the D_0 values ranged from 0.89 to 1.04 Gy, and the D_{10} values ranged from 2.01 to 2.29 Gy. The $\bar{D}$ values ranged from 0.89 to 1.04 Gy. As expected, the AT homozygote strains (GM02052 and AG03058) were markedly radiosensitive with SF_2 values of 0.013 and 0.021, D_0 values of 0.45 and 0.43 Gy, D_{10} values of 1.08 and 1.33 Gy, and $\bar{D}$ values of 0.47 and 0.63 Gy.

The SF_2 values for the various fibroblast strains used in this study, as well as those for 24 normal strains previously reported from our laboratory (16), are shown in Fig. 2. As can be seen, the SF_2 values from the five control strains used in the present study are distributed within the range of the 24 normal strains previously reported, while SF_2 values from the AT heterozygote strains and the patients with severe reactions to radiotherapy are at the lower range of normal. The SF_2 value from the homozygote patients are significantly lower.

The effect of multiple 2.0 Gy fractions separated by 24-h intervals in the various fibroblast strains is shown in Fig. 3. There is a gradual linear reduction in the surviving fraction *versus* total dose of radiation in the control strains, whereas there is a much steeper reduction in the surviving fraction *versus* total dose both in the group of patients who had severe responses to radiotherapy and in the AT heterozygote strains. At 12 Gy total dose (6 fractions of 2.0 Gy), the mean of the surviving fraction of the control group is significantly different from that of the mean of the group of patients with severe responses ($0.0246 \pm .0064$ *versus* $0.00099 \pm .0002$, $P = 0.0002$, Student's t test) and that of the mean of the AT heterozygotes ($0.0246 \pm .0064$ *versus* $0.00109 \pm .0005$, $P = 0.0009$, Student's t test). At 12 Gy total dose, the means of the surviving fractions of the AT heterozygotes and the group of patients with severe responses to radiotherapy are not statistically different ($0.00109 \pm .0005$ *versus* $0.00099 \pm .0002$, $P = 0.7$, Student's t test). The *two solid lines* in Fig. 3 represent the data from the two AT homozygote strains (GM02052 and AG03058) given multiple daily fractions of 0.5 Gy. The individual values for the surviving fraction at 12 Gy taken from the fractionation survival curves shown in Fig. 3 are tabulated in Table 2.

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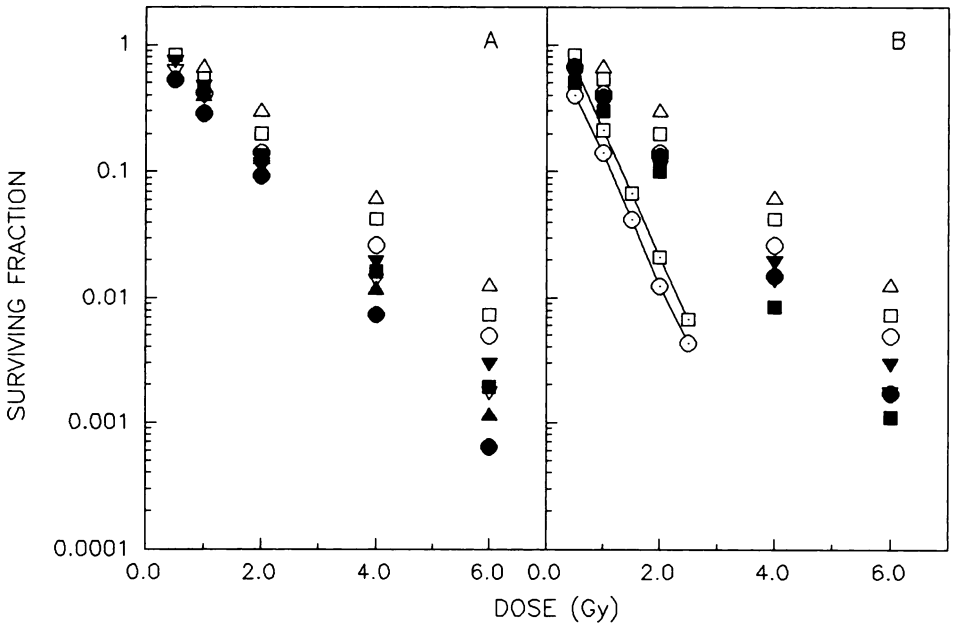

Fig. 1 Single-dose, graded survival curves of fibroblast cells irradiated in the plateau phase and subcultured to low density immediately thereafter. *A*, cell strains from control group: AG01522 (△), RMP-2 (○), RMP-3 (▽), and JL-10 (□). Cell strains from patients with unusually sensitive responses to radiotherapy: JL-7 (●), JL-9 (▼), GB-1 (■), and PO-1 (▲). *B*, cell strains from control group: AG01522 (△), RMP-2 (○), RMP-3 (▽), JL-10 (□). Cell strains from AT heterozygotes: GM03396 (●), AG03057 (▼), and AG03059 (■). Cell strains from AT homozygotes: GM02052 (—⊙—) and AG03058 (—⊡—). Data points for each strain are pooled results of two to four separate experiments.

Table 2 Radiobiological parameters of cell strains used in this study[a]

		Single-dose survival parameters						Fractionated dose survival
	SF_2	N	D_0 (Gy)	D_{10} (Gy)	$\bar{D}$ (Gy)	α (Gy^{-1})	β (Gy^{-2})	SF_{12}[b]
Control group								
AG01519	0.290 ± .079	1.62	1.17	3.24	1.53	0.54	0.041	0.019 ± .004
AG01522	0.306 ± .043	1.50	1.26	3.41	1.60	0.52	0.035	0.029 ± .0015
RMP-2	0.154 ± .014	0.89	1.15	2.50	1.11	0.90	0.000	0.026 ± .0018
RMP-3	0.120 ± .005	1.04	0.93	2.17	0.94	1.07	0.000	0.032 ± .0018
JL-10	0.214 ± .025	1.22	1.21	2.93	1.31	0.72	0.017	0.017 ± .0013
	0.217 ± .082							0.025 ± .0064
Patients with sensitive responses								
JL-7	0.093 ± .009	0.99	0.84	1.93	0.82	1.22	0.002	0.00075 ± .00009
JL-9	0.136 ± .027	1.11	0.96	2.29	1.02	0.98	0.000	0.00089 ± .0001
PO-1	0.124 ± .020	1.27	0.86	2.18	0.95	1.01	0.022	0.0011 ± .00007
GB-1	0.148 ± .006	1.32	0.92	2.35	1.03	0.92	0.023	0.0012 ± .0003
	0.125 ± .024							0.00099 ± .0002
AT heterozygotes								
GM03396	0.131 ± .015	1.14	0.94	2.25	0.99	0.98	0.014	0.00056 ± .00004
AG03057	0.132 ± .014	0.89	1.04	2.29	1.02	0.98	0.000	0.0014 ± .0002
AG03059	0.101 ± .007	0.91	0.89	2.01	0.87	1.15	0.000	0.0013 ± .00008
	0.121 ± .018							0.00109 ± .0005
AT homozygotes								
GM02052	0.013 ± .005	1.23	0.45	1.08	0.47	2.07	0.057	
AG03058	0.021 ± .002	2.14	0.43	1.33	0.63	1.22	0.329	

[a] Data derived from two to four separate experiments for each cell strain. Means of SF_2 and SF_{12} for each cell strain and group are ±1 SD.
[b] SF_{12}, surviving fraction at 12 Gy (see Fig. 3).

Discussion

AT is an autosomal recessive syndrome characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, and immune deficiency. Hypersensitivity to ionizing radiation is, however, the established hallmark of this condition. Fortunately, the incidence of AT is rare, and patients with malignant disease can be successfully treated by radiotherapy with a reduction in dose per fraction and total dose (17).

It has been estimated by Swift *et al.* (18) that approximately 1% of the general population is heterozygous for the *AT* gene. These authors reported that AT heterozygotes carry an excess risk of cancer, especially breast cancer; based on their results, as many as 9–18% of breast cancer patients in the

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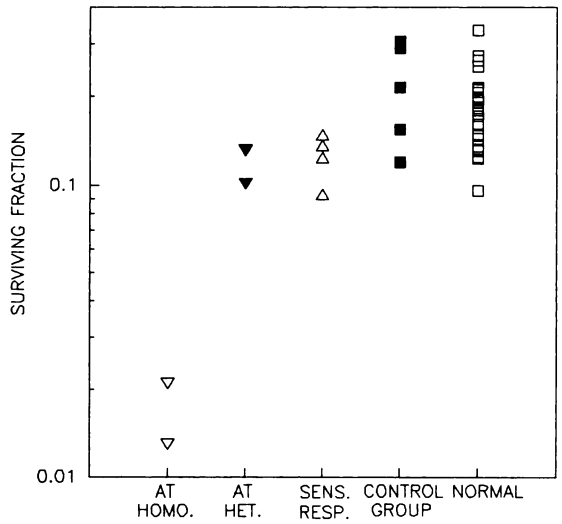

Fig. 2 Radiosensitivity (SF_2 values) from single-dose, graded survival curves of fibroblast cells irradiated in the plateau phase, then immediately subcultured and replated at low density. Data points for each strain are pooled results of two to four separate experiments. The SF_2 values for the normal cell strains are taken from Little *et al.* (16). *Homo*, homozygotes; *Het*, heterozygotes; *Sens. Resp.*, sensitive response.

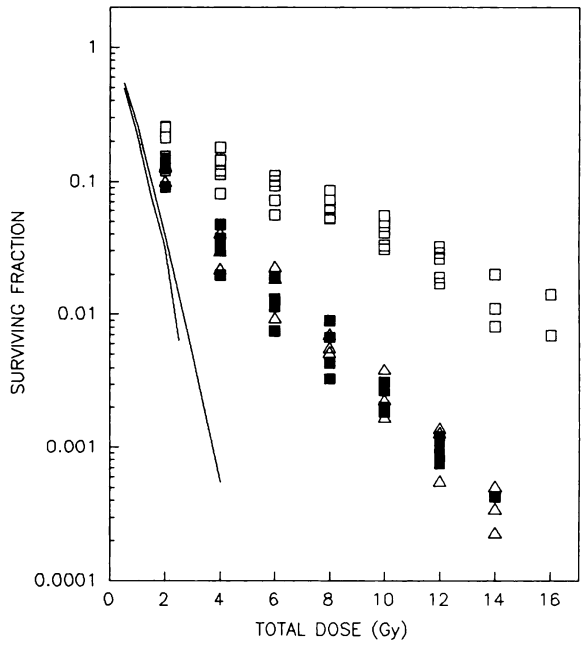

Fig. 3 Fractionation curves of control cell strains (□), strains from patients with sensitive responses to radiation therapy (■), and AT heterozygote strains (△) exposed to multiple fractions of 2 Gy separated by 24-h intervals. Total dose equals the number of fractions × 2 Gy. ——, fractionation curves of the two AT homozygote strains exposed to 0.5 Gy fractions. Results of two to four separate experiments for each cell strain were pooled to obtain data points shown.

United States may be AT heterozygotes. However, these findings remain controversial (19, 20), and final confirmation must await cloning of the *AT* gene when heterozygotes in the general population may be positively identified. AT heterozygotes are asymptomatic and are not usually diagnosed as carriers of the *AT* gene until heterozygote parents bear a homozygote offspring. AT heterozygote fibroblast cell strains are at the lower range of normal in their *in vitro* response to X-irradiation (13, 14). Nagasawa *et al.* (21) proposed the use of an assay based on postirradiation cumulative labeling indexes to discriminate AT heterozygotes from normal cells. This assay is based on the observation that when cells in the plateau phase are irradiated and then subcultured to low density, a fraction of these cells are irreversibly blocked in G_1 and never initiate DNA synthesis. This fraction differs in AT heterozygote and normal cell strains. By use of flow cytometry, Lavin *et al.* (22) reported that AT heterozygotes could be distinguished from controls by the proportion of cultured lymphoblasts in postirradiation G_2 arrest. More recently, Lavin *et al.* (23) used the same assay to demonstrate that breast cancer patients with severe acute reactions to radiotherapy had elevated levels of cells in postirradiation G_2 arrest, a pattern similar to that seen with AT heterozygotes.

Several retrospective studies have shown that dermal fibroblasts derived from patients with unusually sensitive skin responses are radiosensitive *in vitro* (9–11). However, the *in vitro* X-ray survival parameters of fibroblasts derived from such patients generally do not demonstrate the hypersensitive responses seen in AT homozygote fibroblasts. The X-ray survival parameters of non-AT homozygote patients with severe normal tissue clinical responses to radiotherapy are in the lower range seen in fibroblasts from apparently normal cell bank controls, and are similar to the survival parameters of fibroblasts from patients heterozygous for the *AT* gene. However, two isolated cases have been reported in which cells from patients with severe clinical reactions to radiotherapy have demonstrated marked *in vitro* radiosensitivity similar to that seen in cells from AT homozygotes (7, 8). In one of these cases (7), the patient did not show any radiation-related acute or late skin reactions, but instead suffered severe and eventually fatal fibrosis of internal organs in the radiotherapy field. In the other case (8), the patient suffered extreme acute toxicity to radiotherapy and died from late radiation damage. Interestingly, cells from this patient demonstrated a normal delay in DNA synthesis after irradiation.

Investigators have begun recently to use skin and blood samples from cancer patients to try to predict an individual's normal tissue response to radiotherapy (6, 24). It is not clear, however, whether these prospective studies can best predict acute or late severe normal tissue responses (4, 24). Also, in studies comparing the use of skin and blood samples as predictors of patient normal tissue response, there was little evidence for a correlation in the *in vitro* X-ray survival parameters between the dermal fibroblasts and blood lymphocytes (6, 24, 25).

Assays which depend on the surviving fraction from single acute radiation doses may not account for differences in the patient's cells ability to repair radiation-induced DNA damage. In an attempt to discriminate slight differences in the radiation response between normal cell bank controls and

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patients with genetic diseases such as AT, investigators have used chronic low-dose rate exposure to either cesium 137 γ radiation or tritiated water (12, 26, 27). Chronic low-dose rate exposure failed to detect differences in the cell-surviving fraction between AT heterozygotes and normal cell bank controls.

In the present study, we used multiple daily fractions of clinically relevant radiation doses in plateau phase cultures of fibroblasts from patients with severe responses to radiotherapy, AT heterozygote cells, and apparently normal fibroblasts and cell bank controls. We chose this fractionation scheme so as to magnify slight differences in inherent radiation response and because it is similar to clinical fractionation protocols. Two particular findings have emerged. First, AT heterozygotes appear to differ from apparently normal strains in their ability to accumulate and repair radiation damage after exposure to fractionated radiation at clinically relevant doses. Second, fibroblasts from patients who had severe normal tissue reactions after radiotherapy showed a response similar to AT heterozygotes.

It is important to note that the AT heterozygote strains and the fibroblasts from patients with severe normal tissue reactions after radiotherapy were only slightly more sensitive than the control fibroblasts to single-dose irradiation; the SF_2 values are not statistically different. However, after 6 fractions of 2.0 Gy (12 Gy total dose), the mean of the surviving fraction of the control group, which includes one fibroblast derived from a patient that had a normal response to radiotherapy, is markedly different from that of the AT heterozygotes ($P = 0.0009$) and the sensitive response group ($P = 0.0002$; Table 2). There was no apparent difference between the three patients with severe acute responses to radiotherapy and the one patient with the severe late effects of radiotherapy. It is interesting to note that, with the exception of the AT homozygote strains, all of the fibroblast strains used in this study are proficient in the repair of potentially lethal damage (data not shown).

Swift *et al.* (18) estimates that as many as 4.7% of cancer patients between the ages of 20 and 79 years may be carriers of the *AT* gene. It is tempting to speculate that these AT heterozygote patients, who are asymptomatic but may be at excess cancer risk, might be responsible for the unusually sensitive responses seen in clinical radiotherapy. On the other hand, some of the moderately sensitive fibroblast strains from so-called apparently normal individuals (those at the bottom of the range shown in Fig. 2) could be carriers of the *AT* gene.

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