Evidence of DNA Repair/Processing Defects in Cultured Skin Fibroblasts from Breast Cancer Patients

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ABSTRACT

Cultured skin fibroblasts from 14 breast cancer (BC) patients were compared with those from 8 healthy subjects and 4 ataxia-telangiectasia (A-T) cases for sensitivity to low dose-rate (0.007 Gy/min) γ-irradiation assessed by a colony-forming assay and for postirradiation DNA synthesis inhibition determined by the method of [3H]thymidine incorporation. Fibroblasts from all but two BC patients exhibited moderately enhanced radiosensitivity in the colony-forming assay, occupying an intermediate position between the controls and the A-T cases. Fibroblasts from the radiosensitive BC patients also showed an intermediate response with respect to radio-induced DNA synthesis inhibition compared with those from controls and A-T cases. In a host cell reactivation assay using an irradiated herpes simplex virus for plaque-forming ability, the fibroblasts from 7 BC patients, used as host cells, resulted in a significantly reduced infectivity compared to the unirradiated counterparts in the respective cell strains. Defects in DNA repair and/or DNA processing after exposure to genotoxic agents would lead to genomic instability and hence would be responsible for cancer predisposition. Our data suggest that most BC patients may carry various genes resulting in such defects, and additional studies on normal cells from a larger cohort of BC patients and their family members are warranted to establish a connection between mutations or polymorphisms in specific DNA repair genes and susceptibility to breast cancer.

INTRODUCTION

Studies on inherited cancer-prone disorders like A-T and xeroderma pigmentosum suggested a link between cancer susceptibility and radiosensitivity resulting from a deficiency in DNA repair and/or cell cycle deregulation (1, 2). This notion is indeed supported by the later observations that most of the cancer susceptibility genes identified to date result in an impairment of DNA repair and cell cycle processes that may increase genomic instability, leading to carcinogenesis (3–5). The assumption that a considerable fraction of the population may be carriers of various genes increasing their risks of developing cancer led to a number of studies examining normal body cells from cancer patients for abnormal responses to irradiation to verify the connection between radiosensitivity and cancer predisposition (6–10). A high frequency of radiosensitive cases was found among BC patients by cytogenetic assays detecting radio-induced chromatid breaks in G2-phase and micronuclei in G0-phase blood lymphocytes (8, 9). An increase in both of these types of chromosomal aberrations has been attributed to DNA repair deficiency without any further analysis of the repair defects per se in these patients. The micronuclei assay, however, often fails to correlate with cellular response to irradiation as measured by the CFA, a gold standard for assessing radiosensitivity and DNA repair deficiency (11). Also, Scott et al. (12) found a lack of correlation between results obtained by the G2 and the G0 chromosomal assay in the same BC patients, although both of these assays should detect the evidence of DNA repair deficiencies. A deficiency in DNA strand break rejoining has been observed in blood lymphocytes from BC patients, but its correlation with cellular radiosensitivity was not examined in the same patients (13). Thus, the use of any of these methods alone may not be adequate in characterizing all the existing radiosensitive cases in a given population of BC (or other cancer) patients. In earlier studies, using a clonogenic (fibroblast) survival assay after low dose-rate γ-irradiation, we detected enhanced radiosensitivity in most of the patients with NHL and papillary thyroid cancers, many of whom also showed a less than normal level of radio-induced inhibition of DNA synthesis (7, 10). Moreover, radiosensitive fibroblasts from NHL patients were found to be deficient in the repair of DNA double-strand breaks (14) and to show increased chromosomal aberrations after irradiation (15). These data showed an association of cellular/chromosomal radiosensitivity and DNA repair defects in cancer patients, suggesting a connection between such defects and cancer susceptibility.

In the present studies, we examined cultured skin fibroblasts from 14 BC patients, 8 healthy subjects, and 2 A-T patients to compare their survival as assessed by the CFA after low-dose rate γ-irradiation. The same cells were also compared for the levels of radio-induced inhibition of DNA synthesis and their DNA repair capabilities. The data obtained in these studies showed evidence of defects in DNA repair/processing in all the BC patients characterized as radiosensitive by the CFA alone.

MATERIALS AND METHODS

Cells and Cell Survival Assay

Fibroblast strains were developed (upon informed consent) from gluteal or leg skin biopsies obtained from 14 BC patients (all female; age range, 26–60 years), 8 healthy subjects (4 males and 4 females; age range, 29–67 years), and 4 A-T homozygotes (2 males and 2 females; age range, 4–12 years). The CFA was performed as described previously (14, 16). The cell strains were compared for their survival as assessed by the CFA after low-dose rate γ-irradiation (0.007 Gy/min) and incubated overnight at 37°C in a humidified atmosphere with 5% CO2; 95% air. Cells were then trypsinized, harvested, washed, and resuspended in fresh medium. Aliquots from appropriate dilutions of the cell suspensions were assayed (in triplicates at each dose point) for colony-forming ability in dishes containing Ham’s F-12 medium with appropriate supplements were exposed to γ-irradiation (Co60; International Neutronics) at a dose rate of 0.007 Gy/min and incubated overnight at 37°C in a humidified atmosphere with 5% CO2; 95% air. Cells were then trypsinized, harvested, washed, and resuspended in fresh medium. Aliquots from appropriate dilutions of the cell suspensions were assayed (in triplicates at each dose point) for colony-forming ability in dishes containing Ham’s F-12 medium and a feeder layer consisting of (50 Gy) γ-radiation-inactivated normal human fibroblasts (60,000/dish). After 2–3 weeks of incubation with a weekly change of medium, colonies (>50 cells) were stained, washed, and counted. Cell survival curves were drawn based on the (percent) colony counts after a given dose of radiation compared to those in the unirradiated counterparts in the respective cell strains. Each cell strain was analyzed in three or more independent CFAs to calculate...
the mean survival data. All cell survival curves were analyzed by the method of Tarone et al. (16) to determine the relative radiosensitivity of the cell strains by comparing their (mean) \( D_{37} \) values (radiation dose resulting in 37% survival). The \( D_{37} \) values (range) for different groups of cell strains were derived by fitting the model to the data, and a \( t \) test was used to make the formal comparison between the groups for determining the significance of difference in radiosensitivity.

**Estimation of Postirradiation DNA Synthesis**

The levels of radio-induced inhibition of DNA synthesis (high in normal cells and low in cells carrying cell cycle defects such as A-T) were measured by using the method of \( ^{3} \text{H} \)-thymidine uptake (17). Confluent cells were harvested and plated in fresh medium at a density of \( 2 \times 10^{5} \) cells/35-mm dish. After incubation for 24 h, two dishes representing each cell strain were taken out on ice; one was exposed to 4 or 8 Gy of \( \gamma \)-rays (8 Gy/min), whereas the other (unirradiated) served as the control. Both dishes were incubated for another 30 min at 37°C, and then \( ^{3} \text{H} \)-thymidine was added to each (5 \( \mu \text{Ci/dish}; \) specific activity, 5 Ci/mm). After a 2-h incubation, cells from each dish were washed three times with PBS, trypsinized, harvested onto glass fiber filters using a skatron harvester, and then counted for radioactivity in a liquid scintillation spectrometer (higher counts indicated greater DNA synthesis). Inhibition of DNA synthesis was expressed as a percentage of counts/minute in irradiated cells compared to the respective (unirradiated) control for each cell strain. To minimize handling errors, each experiment was run in triplicates for each cell strain, using A-T cells every time as the positive control (for radiosensitive DNA synthesis), and the data were averaged from at least two independent experiments.

**Assessment of DNA Repair Capability**

**HCR.** The HCR method was similar to that described by Abrahams et al. (18). This method has been used to determine DNA repair capabilities of cultured cells (used as hosts) derived from various cancer-prone disorders and known repair-deficient patients (19). In our studies, HSV strain F was used for the infectious center assay, and Vero (African green monkey kidney) cells were used for viral propagation (both HSV and Vero cells were from American Type Culture Collection). Each monolayer of cultured fibroblasts in a 35-mm dish representing a healthy subject or a BC patient was infected with irradiated (500 and 1000 Gy) or unirradiated HSV (in PBS + 2% fetal bovine serum, 200 \( \mu \text{l} \)) and incubated for 90 min with intermittent moving of the inoculum to facilitate adsorption of the virus. Then, 1 ml of growth medium (Ham's F-12) containing 2% serum was added to the dish. After a further incubation for 90 min, the inoculum was removed, and the monolayer was washed with PBS and trypsinized, and cells were harvested and mixed with Vero cells and then seeded into 60-mm dishes containing Eagle's MEM + 10% fetal bovine serum. These dishes were incubated for 5 h, and then growth medium was removed, and an agar (0.45%) overlay was added to each dish. The dishes were incubated for 3–4 days after the agar solidified, and then the plaques were counted in each dish to determine the survival of the virus. The percentage of survival of the virus treated with different doses of radiation and inoculated into a host cell strain was calculated by comparing the number of plaques resulting from the irradiated and the unirradiated virus inoculated into the same host. Student's \( t \) test was used to evaluate the difference in the mean percentage of survival of the virus at each radiation dose level in the two groups of host cells (normal subjects and BC patients). In these experiments, nine of the fibroblast strains from BC patients showing normal response were compared with those from eight healthy subjects for HCR.

**PLDR.** Fibroblast cells (from healthy subjects and BC patients) grown to confluence were irradiated with 4 Gy of \( \gamma \)-rays (0.007 Gy/min). Cells were either harvested immediately (for immediate plating) or after 6, 12, and 24 h of incubation after irradiation (for delayed plating) and analyzed by the CFA to determine the difference in survival. The lack of an increase in survival due to delayed plating of the irradiated cells indicated a deficiency in the repair of potentially lethal damage.

**RESULTS**

Fig. 1 illustrates typical survival curves obtained after low-dose rate \( \gamma \)-irradiation of cultured fibroblasts from healthy subjects, BC patients, and A-T homozygotes. Although these data represent the eight controls and only three BC and two A-T cases, they include the most radiosensitive and radioresistant cell strains of each group, except for two strains from BC patients that fell closer to the healthy fibroblasts for survival. Survival curves for all the cell strains from each group were plotted in the same manner to be analyzed by the statistical approach of Tarone et al. (16), fitting a log-linear model to the data to calculate the \( D_{37} \) value (mean) of each cell strain. The ranges of \( D_{37} \) values (in Gy) thus derived were 3.3–5.1 for 8 controls, 0.3–2.8 for 12 BC cases, and 0.5–1.0 for 4 A-T cases, whereas fibroblasts from the 2 BC patients, which showed survival overlapping with that (range) of the normal subjects, had calculated \( D_{37} \) values of 3.0 and 3.2 Gy. According to the nature of the survival curves and the calculated \( D_{37} \) values, fibroblasts from 12 of the 14 BC patients were found to show significantly enhanced sensitivity to irradiation compared to the normal subjects (\( P < 0.00001 \)), occupying an intermediate position between the normal subjects and the A-T cases. The levels of postirradiation incorporation of \( ^{3} \text{H} \)-thymidine indicating the differences in radio-induced inhibition of DNA synthesis in 3 normal, 3 BC, and 1 A-T fibroblasts are shown in Fig. 2, whereas the ranges of percent DNA synthesis inhibition determined by the same criterion in all the cell strains, after 4 Gy of irradiation, are given in Table 1. These data suggest that the radiosensitive fibroblasts from the BC patients were moderately resistant to radio-induced DNA synthesis inhibition compared to the normal subjects, whereas a few of the BC cases overlapped with A-T cases for this property. However, the 2 BC fibroblast strains with normal survival response showed normal levels of radioinduced inhibition of DNA synthesis. The HCR data in Fig. 3 show the plaque-forming ability (in percent) of the irradiated HSV that infected host cells representing normal or BC fibroblasts. It was noted...
that seven of the nine BC radiosensitive fibroblast strains, tested as hosts, resulted in significantly (P < 0.0001) less recovery of the irradiated virus (reduced survival and hence less plaque formation) compared to the host fibroblasts from 8 normal subjects. Two BC fibroblast strains that exhibited enhanced radiosensitivity in survival curve analysis overlapped with the normal subjects for viral reactivation at the lower dose (500 Gy) of radiation and were not included in the statistical analysis. Also, the fibroblast strain from a BC patient showing normal survival after irradiation was found to be normal for HCR. A reduction in viral reactivation in the seven radiosensitive BC fibroblast cell strains indicated a deficiency in these cells for repairing \( \gamma \)-ray-induced DNA damage in the virus.

For an analysis of PLDR, fibroblasts from six BC patients (found to be radiosensitive in cell survival assay and deficient in HCR) were first compared to those from two normal subjects for survival in the CFA after immediate and delayed (24 h) plating after 4 Gy of high-dose rate irradiation and hence decided to use 4 Gy of high-dose rate irradiation for all the experiments reported here for rapid screening and avoiding an engagement of the radiation source for a long time.

Table 1  
<table>
<thead>
<tr>
<th>Fibroblast source</th>
<th>No. of cell strains analyzed</th>
<th>Range of DNA synthesis inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td>8</td>
<td>46.0–53.0</td>
</tr>
<tr>
<td>Breast cancer patients</td>
<td>2</td>
<td>48.0–58.0</td>
</tr>
<tr>
<td>(Normal survival)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radioactive patients</td>
<td>12</td>
<td>10.0–32.0</td>
</tr>
<tr>
<td>A-T homozygotes</td>
<td>4</td>
<td>7.0–11.0</td>
</tr>
</tbody>
</table>

* We observed qualitatively similar results in several cell strains after high-dose rate and low-dose rate irradiation and hence decided to use 4 Gy of high-dose rate irradiation for all the experiments reported here for rapid screening and avoiding an engagement of the radiation source for a long time.

no improvement in the survival of radiosensitive BC fibroblasts (data representing two normal and four BC strains are shown). Fibroblasts from the two BC patients showing normal survival response were also studied and found to show an improvement in survival on delayed plating (data not shown). Once again, the PLDR data suggested a deficiency in DNA repair capability of the cells from the BC patients who exhibited enhanced radiosensitivity in cell survival analysis.

DISCUSSION

Defects in DNA damage repair and/or DNA processing after exposure to genotoxic agents would lead to genomic instability and in turn to increased susceptibility to malignancies (1–5). Carriers of cancer susceptibility genes are therefore expected to show abnormal
responses to agents like radiation in their body cells. In the present study, at least 12 of 14 BC patients (>85%) were characterized as moderately radiosensitive by the clonogenic survival assay. The frequency of radiosensitive cases among BC patients detectable by CFA appeared to be much higher than that found by other investigators using either G0 micronucleus or G2 chromosomal assays which, used separately, identified 30–50% of the Western BC patients as radiosensitive (8, 9). However, when these two assays were used in the same patients, they yielded different results, i.e., a case detected as radiosensitive by the G0 assay was not found to be radiosensitive by the G2 assay, and vice versa. Scott et al. (12) attributed this difference to DNA repair deficiencies involving pathways/mechanisms that are different for micronuclei formation and G2 chromosomal aberration. If the results of the two assays were combined (12), one would find almost 70% of the BC patients to be radiosensitive, a frequency much closer to what we have found with the CFA. Unfortunately, however, the micronuclei assay often fails to correlate with cellular radiosensitivity (11) and hence is not considered to be very useful in determining radiobiological response, particularly cell death after irradiation. This led us to use the CFA for analyzing radiosensitivity in the fibroblast strains from the BC patients.

Interestingly, most of the radiosensitive cell strains from BC patients characterized as radiosensitive by the CFA in our studies also showed a reduced viral recovery from radio-induced (lethal) damage as determined by the HCR assay. The HCR data suggested that body cells from radiosensitive BC patients carry defect(s) compromising their capability to repair DNA damage. A lack of PLDR after delayed plating of these cells after γ-irradiation provided further evidence of a DNA repair deficiency in the fibroblasts from the BC patients, although the nature of such a defect has yet to be elucidated. Also intriguing was the observation that fibroblasts from most of the BC patients exhibited a moderate resistance to radioinduction of DNA synthesis inhibition, which may indicate a connection between cellular radiosensitivity and a common defect(s) affecting DNA repair and DNA processing after irradiation in these cases. Using the same assays, we made similar observations earlier in cultured fibroblasts from a majority of the patients with NHL and papillary thyroid cancer (7, 10). Those and the present findings would suggest that many cancer patients may be carriers of defective genes/functions that compromise both DNA repair and postirradiation DNA processing (possibly cell cycle regulation) in their normal body cells. These genes may belong to the family of those responsible for cancer susceptibility. Indeed, most of the cancer susceptibility genes discovered to date have been connected directly or indirectly with DNA repair and/or cell cycle checkpoint defects (3–5). Thus, enhanced radiosensitivity resulting from DNA repair defects and/or an anomaly in processing of damaged DNA observed in the BC patients could be responsible for the genomic instability that led to their susceptibility to the malignant disease. Our data were obtained with fibroblasts derived from randomly selected patients prior to therapy and were basically similar to the cytogenetic data in the Western population. Hence, it is very likely that enhanced radiosensitivity is a general feature of the BC patients and not specific for the cohort of patients studied.

The literature does indicate that genes affecting cell cycle regulation or DNA repair may be structurally or functionally altered in BC patients. Birrell and Ramsay (20) noted a reduction in the radioinduction of the tumor suppressor protein p53 in lymphoblastoid cells from 18% of the BC patients, similar to what was observed in A-T heterozygotes. The levels of radioinduced p53 were not correlated with other radiobiological abnormalities in these patients. Using the Western blot technique, we observed either a reduced induction or no induction of p53 after γ-irradiation4 in at least 4 of the 10 (40%) fibroblast strains from our BC patients, which also exhibited enhanced sensitivity to low-dose rate irradiation and less than normal inhibition of DNA synthesis. These data indicated that the defects carried by the BC patients (DNA repair-processing) may involve p53 regulation in some cases. Price et al. (21) reported rare microsatellite polymorphisms in the DNA repair genes in cancer patients of varying radiosensitivity. Similar studies on the radiosensitive cell strains from BC patients and their asymptomatic family members may lead to the identification of alterations in specific DNA repair genes accounting for our observations and establishing their links to cancer susceptibility. In addition, the BC patients could be carriers of genes like BRCA-1/BRCA-2 (associated with familial breast cancer) or A-T (18% of BC patients are believed to be A-T heterozygotes) which have a functional link, and are known to participate in a network of pathways including the ones controlled by p53 affecting DNA repair/processing defects 

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REFERENCES


4 Unpublished observations.


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