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 (P < 0.0001) recovery of the virus relative to the 8 control fibroblasts, suggesting a deficiency in DNA repair in the former. A number of the BC fibroblasts analyzed in an assay for potentially lethal damage repair confirmed the repair deficiency in the fibroblasts from the BC patients. 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 glutural 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 used to determine cell survival after irradiation has been described (7, 10). Briefly, fibroblast cells (passages 3–12) grown to confluence in tissue culture 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) D37 values (radiation dose resulting in 37% survival). The D37 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 [3H]thymidine uptake (17). Confuent cells were harvested and plated in fresh medium at a density of 2 × 10^6 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 γ-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 [3H]thymidine was added to each (5 μCi/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 per milligram protein from the experimental dish compared with controls. The cells were then harvested into 10% trichloroacetic acid (tca) in a scintillation vial, and the radioactivity was determined using a Beckman scintillation counter.

In order to study the radio-induced inhibition of DNA synthesis in human fibroblasts from patients with BC and normal individuals, the fibroblasts were infected with HSV at a concentration of 10^4 plaque-forming units per dish. The virus was harvested 48 h after infection, and the cell monolayers were exposed to γ-irradiation (1 Gy) before using for radio-induced inhibition of DNA synthesis.

RESULTS

Fig. 1 illustrates typical survival curves obtained after low-dose rate γ-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 D37 value (mean) of each cell strain. The ranges of D37 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 D37 values of 3.0 and 3.2 Gy. According to the nature of the survival curves and the calculated D37 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 [3H]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

**PLDR.** Fibroblast cells (from healthy subjects and BC patients) grown to confluence were irradiated with 4 Gy of γ-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.

![Fig. 1. Fibroblast cell survival curves, after low-dose rate irradiation, of eight healthy subjects ( ), three BC patients ( ), and two A-T homozygotes (■ and ▪). Each cell strain was analyzed in two to four independent experiments (bar; SE). These survival curves include the most radiosensitive and radioresistant cell strains of each group (normal, BC, and A-T) and are typical of those observed with all cell strains in each group for which D37 values are given in the text. The shaded lines between [ ] represent the data (range) for eight normal subjects, each of which was analyzed 2–4 times; error bars are not shown for clarity.](image-url)
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 for all the experiments reported here for rapid screening and avoiding an engagement of the radiation source for a long time.

Table 1  Levels of radiation (4 Gy)$^a$-induced DNA synthesis inhibition determined by the $[^3H]$thymidine uptake method in fibroblasts from healthy subjects, BC patients, and A-T homozygotes

<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>Normal survival</td>
<td>2</td>
<td>48.0–58.0</td>
</tr>
<tr>
<td>Breast cancer patients</td>
<td>12</td>
<td>10.0–32.0</td>
</tr>
<tr>
<td>Normal survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiosensitive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast cancer patients</td>
<td>4</td>
<td>7.0–11.0</td>
</tr>
<tr>
<td>A-T homozygotes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very radiosensitive</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ 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 G0/1 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 G0/1 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 micronucleus 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 cultured fibroblasts 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-irradiation 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 BRCA1/BRCA2 (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 (20, 22). It is unlikely that a single gene would account for the varying radiosensitivity and other abnormal cellular responses to irradiation in all the BC patients. Because a surprisingly high frequency of the patients with sporadic BC in different populations appears to be radiosensitive in both cytogenetic and clonogenic survival assays, it is likely that more than one gene sharing common phenotypes, i.e., deficiency in DNA repair/processing, would account for the radiobiological abnormalities and cancer susceptibility in these patients.

Genes for cancer susceptibility have been identified, and their properties have been characterized by studies on inherited cancer-prone disorders and familial cancers (3–5) that represent only a small fraction of the population of cancer patients. The role of such genes in sporadic cancers, comprising most cancer patients, has been implied but remains speculative without a direct demonstration of abnormal responses to carcinogens in the somatic cells of these patients (i.e., DNA repair/cell cycle defects, genomic instability, metabolic anomalies, and so forth). The data obtained with body cells from BC patients in different laboratories including ours strongly suggest a connection between the properties linked to cancer susceptibility genes and the origin of sporadic cancers. In addition, the cancer susceptibility genes may affect the normal or tumor cell response in patients undergoing radiotherapy. These possibilities underscore the need for development of rapid DNA repair/radiosensitivity assays to predict the normal tissue (early or late) and tumor cell response to radiotherapy in BC patients, as well as to screen potentially cancer-prone individuals.

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REFERENCES


4 Unpublished observations.

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