Combined Immunodeficiency Associated with Increased Apoptosis of Lymphocytes and Radiosensitivity of Fibroblasts

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ABSTRACT

Severe immunodeficiency characterized by lymphopenia was found in two siblings, one of whom was examined in detail. The calcium flux, pattern of tyrosine phosphorylation of proteins, and interleukin 2 (IL-2) production and proliferation in response to mitogens suggested that the peripheral blood T cells activated normally. The peripheral blood T cells were shown to have an activated phenotype with increased expression of CD45RO+ and CD95/Fas. Increased spontaneous apoptosis occurred in unstimulated lymphocyte cultures. The elevated apoptosis was not due to alterations in expression or to mutations in Bcl-2, Bcl-XL, or Flip, nor could the spontaneous apoptosis be prevented by blocking Fas, suggesting that it was independent of Fas signaling. This is the first inherited combined immunodeficiency associated with impaired lymphocyte survival.

Fibroblasts derived from the patient showed appreciable radiosensitivity in clonal assays, but apoptosis was not elevated. Our results show that the fibroblasts represent a new radiosensitive phenotype not associated with combined immunodeficiency associated with impaired lymphocyte survival.

INTRODUCTION

To maintain homeostasis, the survival and death of an array of differentiated cells in multicellular organisms must be carefully balanced. A physiological process of cell suicide, known as apoptosis, occurs ubiquitously and serves to remove unwanted cells (1). The decision to proliferate or undergo programmed cell death is dependent upon a delicate balance between intrinsic and extrinsic triggers for cell death and those for cell survival. In the immune system, ensuring that only appropriate lymphocytes survive is of utmost importance. Lymphocytes die if they are not provided with adequate stimulation by antigen, costimulation, and/or cytokines, a concept that has been termed “death by neglect” (2). Alternatively, they can die as a result of repeated antigenic stimulation or in response to self-antigen (2).

Bcl-2 and a related protein, Bcl-XL, and growth factors such as IL-3, IL-6, and IL-7 play important roles in the prevention of the spontaneous apoptosis of lymphocytes (3–6). Cell death associated with antigenic stimulation, on the other hand, cannot be prevented by activation of survival-associated genes such as Bcl-2 but appears to involve a separate pathway that is transduced through CD95/Fas, a member of the TNF-receptor family, and related proteins (7–9).

It is likely that mutations in genes controlling apoptosis in lymphocytes could give rise to immunodeficiency. Although this has not been reported previously, derangements in the balance between apoptosis and cell survival have been suggested to contribute to the pathogenesis of a variety of human diseases such as neoplasia, viral infections, neurodegenerative diseases, and AIDS (10–12), and studies of Bcl-2 knockout mice have shown that, although lymphocytes mature normally, they undergo massive apoptosis in the periphery, with numbers dropping off rapidly as the animal ages (13).

Recent studies have demonstrated an association between immune deficiency and radiosensitivity. A-T, T−, SCID (13–15) and NBS are hereditary disorders associated with diverse clinical features including immune deficiency and clinical and cellular radiosensitivity (14–17). Cell lines from A-T and NBS patients are unable to arrest at cell cycle checkpoints after irradiation, suggesting that they may have defects in checkpoint control. They also, however, have defects in mechanisms that repair the damage induced by ionizing radiation (18, 19). The study of cultured radiosensitive rodent cell lines has demonstrated that defects in DNA DSB rejoining are frequently coupled with an inability to carry out V(D)J recombination (20). Genes operating in this pathway have been identified and include KU70, KU80, DNA-PKcs, and XRCC4 (21), and mice harboring such defects display a SCID phenotype (22, 23). Radiosensitive cell lines derived from SCID patients have been described, but surprisingly, the majority of them are proficient at DSB rejoining (14). Collectively, these results demonstrate a significant overlap between immune deficiency and radiosensitivity, suggesting the common usage of a number of gene products that possibly operate in different damage response mechanisms.

We observed that the lymphocytes of an infant with immunodeficiency characterized by lymphopenia displayed elevated apoptosis in vitro and that the infant’s fibroblasts were radiosensitive. We therefore undertook a detailed investigation of the cells from this infant. Our results suggest that the underlying defect may exhibit different outcomes in different cell types and that there may be a common gene product influencing both radiosensitivity and the apoptotic response.

MATERIALS AND METHODS

Case Reports. The first and third child of an unrelated couple were similarly affected by a severe immunodeficiency. The first child suffered from recurrent upper and lower respiratory infections from the age of 6 months and became significantly unwell from the age of 4 years. She was investigated for immunodeficiency at 8 years, by which time she had poor growth, chronic sinusitis and serous otitis media, extensive warts, recurrent lower respiratory infection with bronchiectasis, intestinal candidiasis, and severe protracted diarrhea due to cryptosporidium. She showed an absolute lymphopenia of T and B cells (Table 1); T cells displayed evidence of activation (CD3+HLA-DR+), 26%. There were no detectable tonsils or thymus. She was hypogammaglobulinemic [IgG, 3.81 g/l (NR for age, 7.0–12.6); IgA, <0.07 g/l (NR, 3454]
The parents. Short stature (height 1.22 m, median 1.58 m) and hypogammaglobulinemia [IgG, 4.1 g/l (NR, 5.3–10.1); IgA, <2 IU/ml (median, 18)] with no specific antibody responses. Adenosine deaminase and purine nucleoside phosphorylase levels were normal. Chromosome analysis showed a normal XY karyotype. Lymphocyte cultures exposed to 1 Gy γ-irradiation showed 30 lesions/50 cells compared with 16 lesions/40 cells in control cells.

For routine cell survival, cells in the exponentially growing phase were trypsinized and irradiated 4 h after plating. Colonies were fixed and stained after 9–12 days of incubation. RPLD experiments were carried out, as described previously (25). In brief, cells maintained in 0.5% serum for 5 days were irradiated and either maintained for an additional 5 days in 0.5% serum prior to plating to determine survival (delayed plating samples) or immediately trypsinized and plated at low density to determine survival (immediate plating samples).

Sequencing of cDNA. Total RNA, extracted from EBV-transformed B cells, was used to synthesize cDNA by incubating with oligo-dTs (Life Technologies, Gaithersburg, MD) and SUPERSCRIPT II RNase H reverse transcriptase according to the manufacturer’s instructions (Life Technologies). PCR of cDNA with Taq polymerase (Perkins Elmer, Branchburg, NJ) for Bcl-2, FLIP or Advantage-GC cDNA PCR kit for Bcl-2 and Bcl-X (27) and whole-cell extracts from fibroblasts (28) were prepared as described previously. EMSA was carried out with a γ-32P-labeled double-stranded oligonucleotide M1/M2, and DNA-PK activity was analyzed by measuring the phosphorylation of a p53-derived peptide (29) with modifications also described previously (30). Western blotting was carried out as described previously (30). The anti-XRCC4 and anti-DNA-PKcs antibodies used were 6J4 and 18-2 (31). Antibodies to p55, hMrEl1, and hRAd20 were a kind gift from Dr. J. Petriini (University of Wisconsin Medical School, Madison, WI) (32, 33).

Analysis of Intracellular Bcl-2 Expression. Increased susceptibility to apoptosis has been associated with low expression of Bcl-2 (5, 34), which can be improved by the addition of IL-2. Analysis of intracellular Bcl-2 expression was performed with the following intracellular staining procedure. PBLs (1 × 10^6) were incubated in FACS Lysing Solution (Becton Dickinson) to lyse any remaining RBCs. The cells were then pelleted and incubated in FACS Permeabilizing Solution (Becton Dickinson) before being washed in wash buffer (PBS, 0.5% BSA and 0.1% NaN₃) and incubated in 20 μl FITC-conjugated anti-human Bcl-2 mAb (IgG1, 100 μg/l; DAKO A/S, Glostrup, Denmark) or mouse IgG1 (as an irrelevant control). The cells were washed again in wash buffer and then fixed with 1% paraformaldehyde before analysis on FACSscan.

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### Table 1: Phenotype of the patient’s and his sister’s lymphocytes

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>Patient</th>
<th>Sister</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td>1100</td>
<td>1700</td>
<td>2000–4000</td>
</tr>
<tr>
<td>Phenotype of lymphocytes (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3+</td>
<td>54</td>
<td>44</td>
<td>70–90</td>
</tr>
<tr>
<td>CD4+</td>
<td>30</td>
<td>14</td>
<td>35–65</td>
</tr>
<tr>
<td>CD8</td>
<td>22</td>
<td>38</td>
<td>15–35</td>
</tr>
<tr>
<td>CD16/56+</td>
<td>12</td>
<td>58</td>
<td>1.0–10</td>
</tr>
<tr>
<td>CD19+</td>
<td>32</td>
<td>5</td>
<td>1.0–10</td>
</tr>
<tr>
<td>CD25+</td>
<td>22</td>
<td></td>
<td>0–9</td>
</tr>
<tr>
<td>Phenotype of CD3+ lymphocytes (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA DR+</td>
<td>32</td>
<td>26</td>
<td>1.0–15</td>
</tr>
<tr>
<td>CD45R0</td>
<td>83</td>
<td>ND*</td>
<td>40–60</td>
</tr>
<tr>
<td>CD45RA</td>
<td>10</td>
<td>ND</td>
<td>40–60</td>
</tr>
<tr>
<td>CD95Fas+</td>
<td>100</td>
<td>&lt;10</td>
<td></td>
</tr>
</tbody>
</table>

*ND, not done.*
Measurement of DNA DSBs. The DNA DSB-rejoining protocol used was as described previously (39). Briefly, 2 × 10^6 exponentially growing cells were grown for 4 days in MEM plus 20% FCS before labeling and irradiation. For irradiation, the culture flasks were placed on ice for 30 min before irradiation, and the culture medium was kept cool throughout the period of irradiation.

V(D)J Recombination. V(D)J recombination was assessed, as described by Nicolas et al. (15).

Analysis of Cell Cycle Checkpoint Arrest. The protocol for the measurement of DNA synthesis after radiation (RDS protocol) was followed as described previously (40). The extent of G_1 and G_2 arrest was measured by FACs analysis, as described previously (30).

Micronucleus Formation. Cells were plated at 5 × 10^4 cell/dish, left overnight, and irradiated with the relevant dose. Samples were taken immediately, and cytochalasin B (1 μg/ml) was added to the remainder. Cells were subsequently fixed and stained with 16% Giemsa at daily intervals for 7 days, and micronucleus formation was estimated in binucleate cells. Micronucleus formation plateaued after 48 h, and for the dose response data shown, samples were taken after 3 days.

RESULTS

Examination of the Patient's Lymphocytes

Phenotypic and Functional Analysis. Phenotypic analysis of the lymphocyte subsets showed that the patient and his sister had T-cell lymphopenia (Table 1), which was progressive with age (Fig. 1). T cells showed a normal distribution of TCR γδ+, TCR αβ+, and Vβ subsets, confirming that the cells did not consist of single or multiple abnormal clones (data not shown). There was also no appreciable alteration in the distribution of lymphocyte subtypes (Table 1). A high frequency (80–95%) of the patient’s T cells expressed the CD45RO isoform, a characteristic of memory or primed cells (shown for CD3^+ lymphocytes in Table 1; similar frequencies also found for CD4^+ lymphocytes). Correspondingly, there were few naive cells (5–20%) with the CD45RA^+ isotype. A normal calcium flux and tyrosine phosphorylation of proteins were observed after activation with anti-CD3 mAb, suggesting that the patient’s T cells could be activated normally through the TCR/CD3 complex. IL-2 production measured by ELISA was normal, and the T cells proliferated in response to mitogens (summarized in Table 2).

Elevated Apoptosis of the Patient’s PBLs. The lymphopenia, coupled with the observation that the patient’s cells appeared to die in vitro, led us to examine the cells for apoptosis. Significantly, elevated apoptosis was observed in the patient’s PBLs (Table 3). Similar results were obtained for enriched T (E^+ ) cells. The increase in numbers of apoptotic cells after γ-irradiation was similar to that of a matched control, suggesting that the lymphocytes did not show increased sensitivity to apoptosis after γ-irradiation (Fig. 2).

Analysis of Candidate Defective Genes and Processes. We next analyzed the expression of candidate defective proteins that might be associated with elevated apoptosis, and we sequenced candidate defective genes and examined other responses likely to influence apoptosis. A summary of this analysis is given in Table 2. Data have been shown only when the response was significantly different from that of the control cells.

Neither the protein levels nor sequence of Bcl-2 or Bcl-X_L was altered in the patient’s cells. The addition of IL-2 diminished the spontaneous apoptosis seen in the patient’s cells to the same degree as that observed in control cells (Table 2). Bcl-2 expression on the patient’s cells was normal with or without the addition of IL-2 (Table 2). Signals transduced via Fas/CD95 may engage the cell death machinery, allowing apoptosis to ensue (41, 42). Because all of the patient’s CD3^+ T lymphocytes expressed Fas, we added a blocking anti-Fas mAb (ZB4) to both unstimulated and stimulated cultures, but no change in the spontaneous apoptosis was observed (Table 2). FLIP is an inhibitor of apoptosis induced by Fas and other members of the TNF receptor family (43–45). No mutations were detected in the coding regions of either the short and long forms of FLIP. Finally, we examined whether apoptosis was elevated after stimulation through the CD3 complex and did not observe any increase in apoptosis after 3 days’ culture with immobilized anti-CD3 compared with unstimulated cultures.

Examination of the Patient’s Fibroblasts

Sensitivity to DNA-damaging Agents. A primary skin fibroblast cell line was established from the patient. The fibroblasts grew well with a similar doubling time to that of control cells and showed no evidence of early senescence. The cells, however, displayed significant radiosensitivity compared with control lines (Fig. 3), although the level of sensitivity was less marked than that of a typical A-T cell line included for comparison. No significantly enhanced sensitivity was
observed to other DNA-damaging agents, including UVC and the cross-linking agent mitomycin C (data not shown).

We investigated the patient’s cells using a number of assays characterized previously associated with radiation sensitivity and for the expression of candidate defective proteins (21, 46 – 48). These data are summarized in Table 4. The induction and rejoining of DNA DSBs were similar to those observed in control cells (Fig. 4). The patient’s fibroblasts showed a slightly faster initial rate of rejoining compared with control cells, which is a feature also seen in A-T cells (49). A-T cells, additionally, display a small but reproducible, elevated frequency of unrejoined DSBs (49), which was not observed in the patient’s fibroblasts. The patient’s cells also displayed a normal ability to carry out V(D)J recombination.

The patient’s fibroblasts displayed normal cell cycle checkpoint responses after irradiation, including normal G 1 -S and G 2 -M checkpoint arrest, normal induction of p53 and p21 after 3 Gy irradiation, and normal arrest of DNA synthesis after radiation, a phenotype defective in A-T cells that gives rise to radioresistant DNA synthesis (47, 48). A-T cells were included as controls in these experiments and showed the anticipated defective responses.

Previous studies have shown that when plateau phase mammalian cells are maintained under nongrowing conditions for a period after irradiation, the survival is elevated, demonstrating the operation of a cell cycle checkpoint response called the G 1 -S checkpoint. This response is particularly important for cells that have been damaged by radiation, as it allows them to repair damaged DNA before proceeding into S phase and potentially forming more DNA damage. Cells that are unable to activate this checkpoint response are more likely to undergo apoptosis or DNA fragmentation, leading to cell death.

We next examined the expression of candidate defective proteins in the patient’s cells. Table 2 shows the results of these analyses.

<table>
<thead>
<tr>
<th>Question addressed</th>
<th>Analysis</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-cell function</td>
<td>T cell proliferation measured with tritiated thymidine; IL-2 production measured by ELISA; calcium influx measured by FACS; tyrosine phosphorylation of proteins after activation via CD-3 complex measured by Western blotting</td>
<td>Normal responses</td>
</tr>
<tr>
<td>Expression of Bcl-2 in apoptotic and nonapoptotic cells</td>
<td>Lymphocytes examined by FACS after 36 h in culture</td>
<td>Identical expression to control cells</td>
</tr>
<tr>
<td>Expression of Bcl-2</td>
<td>Cell lysates of E 67 cells analyzed by Western blotting</td>
<td>Normal Bcl-2 protein levels</td>
</tr>
<tr>
<td>Expression of Bcl-X L</td>
<td>Cell lysates of EBV cell line cells analyzed by Western blotting</td>
<td>Normal Bcl-X L protein levels</td>
</tr>
<tr>
<td>Sequence analysis of bcl-2 and bcl-X L</td>
<td>Reverse transcription-PCR</td>
<td>No mutations in the coding regions of bcl-2 or bcl-X L</td>
</tr>
<tr>
<td>Ability of IL-2 to reduce the elevated apoptosis</td>
<td>Apoptosis measured with and without IL-2 present</td>
<td>IL-2 diminished apoptosis to the same level seen in control cells; thus, the elevated apoptosis remained</td>
</tr>
<tr>
<td>Involvement of Fas/CD95 signaling in the apoptotic response</td>
<td>Apoptosis measured in unstimulated and stimulated lymphocytes after treatment with a blocking anti-Fas mAb (ZB4)</td>
<td>No change in apoptosis frequency</td>
</tr>
<tr>
<td>Sequence analysis of FLIP</td>
<td>Short and long forms of FLIP sequenced by RT-PCR</td>
<td>No mutation detected in coding region</td>
</tr>
<tr>
<td>Involvement of CD3 complex in the apoptotic response</td>
<td>Apoptosis examined in unstimulated cells and cells cultured for 3 days with immobilized anti-CD3; apoptosis also examined following addition of IL-2 and ZB4 to the activated cultures</td>
<td>No change in apoptosis frequency</td>
</tr>
</tbody>
</table>

The data from Table 2 indicate that the patient’s cells are able to express and respond to normal levels of the proteins associated with radiation sensitivity, suggesting that the elevated apoptosis observed in patient cells is not due to defects in these pathways. However, further studies are necessary to determine the exact mechanism behind the elevated apoptosis in these cells.
recovery process, termed the repair of potentially lethal damage (25). Neither A-T cells nor DSB repair-defective rodent cells show enhanced survival under such conditions, demonstrating a defect in this recovery process (50–52). Elevated survival of the patient’s cells observed under the delayed plating conditions was similar in magnitude to that observed for the control cells, such that the sensitivity relative to control cells treated in the same way was maintained (Fig. 3).

Although chromosome analysis of the patient’s lymphocytes suggested that they may display slightly elevated spontaneous and radiation-induced chromosome breakage, the aberrations found were within the normal range, and difficulties in culturing the lymphocytes precluded our reaching firm conclusions. The formation of micronuclei after radiation was measured to assess whether the patient’s fibroblasts displayed elevated chromosome breakage. No elevation in micronuclei was observed compared with control cells, suggesting that the patient does not have a chromosome breakage disorder (Table 5).

**Apoptosis in Fibroblasts.** The elevated level of apoptosis observed in the patient’s lymphocytes prompted us to examine whether the fibroblasts derived from this patient were also prone to apoptosis. Although there was a small increase in apoptosis in the patient’s primary fibroblasts after 3 Gy of irradiation, this was not reproducible with higher doses of irradiation (Table 6).

### Table 4 Analysis of the patient’s fibroblasts

<table>
<thead>
<tr>
<th>Question addressed</th>
<th>Analysis</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ability to repair DNA DSBs</td>
<td>Pulsed-field gel electrophoresis after irradiation</td>
<td>Close to normal (Fig. 4)</td>
</tr>
<tr>
<td>Ability to carry out V(D)J recombination</td>
<td>Signal and coding join formation examined using an <em>in vitro</em> plasmid assay</td>
<td>Normal rejoining</td>
</tr>
<tr>
<td>Ability to arrest at cell cycle checkpoints</td>
<td><em>(a)</em> FACS analysis of primary fibroblasts examining G1-S and G2-M arrest</td>
<td>Normal responses</td>
</tr>
<tr>
<td></td>
<td><em>(b)</em> Radiation (RDS) assay</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>(c)</em> p53 and p21 induction by Western blotting</td>
<td></td>
</tr>
<tr>
<td>Repair of potentially lethal damage</td>
<td>Survival examined in cells held in G0 following immediate and delayed plating</td>
<td>Normal recovery (Fig. 3)</td>
</tr>
<tr>
<td>Radiation-induced chromosome breakage</td>
<td>Micronucleus formation examined after irradiation</td>
<td>Normal response</td>
</tr>
<tr>
<td>Apoptotic response</td>
<td>Apoptag method used to estimate apoptosis in untreated and irradiated cells</td>
<td>No detectable apoptosis</td>
</tr>
<tr>
<td>Examination of candidate genes/proteins</td>
<td>DNA end-binding activity and DNA-PK activity were examined; the levels of XRCC4, p95, hMre11, and hRad50 were examined by Western blotting</td>
<td>Normal DNA-PK activities; normal protein levels</td>
</tr>
</tbody>
</table>

### Table 5 Examination of micronucleus formation in patient and control fibroblasts after exposure to ionizing radiation

| Micronuclei (%) |
|-----------------|-----------------|-----------------|-----------------|
| Dose (Gy)       | Control         | Patient         | ATIBR           |
| 0               | 2.7             | 1.2             | 6.5             |
| 0.5             | 4.7             | 6.8             | 20.5            |
| 1               | 13              | 7.8             | 14.5            |
| 2               | 26              | 20.5            | 50.75           |

**EXAMINATION OF POTENTIAL CANDIDATE GENES.**

The patient’s cells had normal levels of double-stranded DNA end-binding activity, as determined by EMSA, and DNA-PK activity, as determined by a phosphorylation assay with a p53-derived peptide (data not shown). The findings indicate that the activity of DNA-PK, which functions in nonhomologous end-joining in mammalian cells, is normal. Normal levels of p95, the protein recently shown to be defective in NBS (32, 53), and hMre11 and hRad50, two proteins that interact with p95, were observed by Western blotting.

**DISCUSSION**

We describe two siblings with combined immunodeficiency associated with marked lymphopenia that was progressive with age. The progressive loss of lymphoid tissue was demonstrable clinically because neither child had any evidence of a thymus on computed tomography scan, tonsillar tissue could not be seen, and only small peripheral lymph nodes were present. Moreover, the sister displayed significant immune dysfunction only in middle childhood, in contrast to most children with fatal inherited immune deficiency, who manifest problems from early infancy. Additionally, unlike other combined immune deficiencies, the T cells seem to be functionally normal. Here, we report increased spontaneous apoptosis of the lymphocytes *in vitro* in one of the siblings (referred to as the patient throughout the report). Our results suggest that a defect in the control of spontaneous apoptosis of immune cells may be the cause of the clinical disorder observed. This represents the first inherited combined immunodeficiency associated with abnormal control of lymphoid cell survival.

Increased spontaneous apoptosis of lymphocytes *in vitro* has been associated with low Bcl-2 expression, which can be abrogated by the addition of IL-2 (34, 54). Bcl-2 knockout mice show a phenotype similar to that observed in our patient with increasing lymphopenia with age and increased susceptibility to apoptosis (13). Primed or memory T lymphocytes that express the CD45RO isoform display increased spontaneous apoptosis *in vitro* associated with decreased...
sensitivity is not associated with a cell-cycle checkpoint defect nor a V(D)J recombination defect and is not a chromosome breakage disorder. Our results, however, do not allow us to conclude unequivocally whether the patient’s fibroblast cell line has a DNA-repair defect or whether it is defective in some other damage-response mechanism.

Our results show that the mutation in this patient lies in a gene that influences both radiosensitivity and a mechanism that commits to apoptosis. p53 plays a role both in cell-cycle checkpoint control and in the control of apoptosis, and the ATM protein, which is defective in A-T, acts upstream of p53 (14, 46, 68). Although a defect in a pathway involving p53 has not been formally excluded, it is unlikely because p53 is induced normally in the patient’s fibroblasts after γ-irradiation, and no cell-cycle defects have been observed. It is intriguing, therefore, that the defect in our patient, like that in A-T, impinges upon a DNA repair or damage-response process and the apoptotic response, and that, at least in certain cell lineages, these appear separable. This kindred demonstrates a unique immune deficiency that combines elevated spontaneous apoptosis in cells of lymphoid origin and radiation sensitivity of the fibroblasts and suggests an overlap between these two responses, either at the mechanistic level or by a shared signal transduction pathway.

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