A Study on the Activity of Fibroblast Cells in Connection with Tissue Recovery in the Wounds of Skin Injury after Whole-body Irradiation

Jifu QU*, Tianmin CHENG, Chunmeng SHI, Yuan LIN and Xinze RAN

Whole-body irradiation/Fibroblasts/Tissue recovery/Impaired wound healing.

The 6 Gy of whole-body irradiation (WBI) with gamma rays results in an impairment of injured skin tissue recovery and renders a delay in the healing process. For an understanding of whether WBI has damaging effects on fibroblasts in wounds, fibroblasts in wounds combined with WBI and those of simple incision were isolated and cultivated, and abilities connected with tissue repair, including proliferation, attachment, adhesion, and apoptosis, were determined by direct cell count, immunohistochemical staining for proliferation cell nuclear antigen (PCNA), and TUNEL assay. The results showed that the abilities of proliferation and the attachment and adhesion of fibroblasts from wounds combined with WBI significantly decreased in comparison with those having simple incisions on the 3rd and 5th days of posttrauma, whereas the apoptotic ratio of fibroblasts from wounds combined with WBI significantly increased. These data suggest that WBI may exert damaging effects on fibroblasts in wounds, which might be one of the dominant reasons for the impaired healing of wounds combined with WBI.

INTRODUCTION

Combined radiation injury is one of the main injuries occurred during nuclear explosions in wartime and nuclear accidents in peacetime. In clinical practice, if the excision of a malignant tumor is followed by radiation therapy, combined radiation injury may occur too. It has been reported that wounds combined with whole-body irradiation (WBI) heal slowly, but the mechanisms are not fully clarified.

Fibroblasts play critical roles in the process of wound repopulation and subsequent remodeling, and they are also the most important kinds of cells participating in tissue repair. It has been reported in our previous study that the quantity of fibroblasts and the formation of granulation tissues in wounds combined with WBI are significantly decreased in comparison with those of simple incision. The contents of growth factors (such as bFGF and NGF) and extracellular matrix (such as fibronectin and type I collagen) in wounds combined with WBI also were significantly decreased. These data suggest that the abnormalities of the quantity and function of fibroblasts in wounds combined with WBI are one of the important reasons why the healing of wounds is significantly impaired. However, fibroblasts were not radiosensitive, and acute wound healing proceeds in a carefully regulated fashion, which is controlled by several kinds of cells, cytokines, and extracellular matrixes. Because of the network interaction of cytokines and fibroblasts, the decrease of cytokines in wounds combined with WBI will inevitably affect the function of fibroblasts and the healing process. Therefore it is not clear whether WBI has damaging effects on fibroblasts in wounds, whether this kind of damage influences the process of tissue repair has not been previously clarified.

In the present study, the fibroblasts in the wounds with WBI as well as simple incisions were isolated to be cultivated in vitro. Their ability connected with tissue recovery was observed for the damaging effects of the WBI with gamma rays, which might be considered to be a possible mechanism of impaired wound healing.

MATERIALS AND METHODS

Twenty Wistar rats, female and male, were used in our study. They were randomly divided into two groups: (1) Simple cutaneous incision group (S group, n = 10); (2) Combined WBI group (C group, n = 10). The rats were 15 weeks old and weighed 200 ± 20 g. The rats of S group were anesthetized by an intraperitoneal injection of pentobarbital (30 mg kg⁻¹). Two linear full-thickness skin incisions were then made, each of which was 6 cm long. The incisions were sutured to close the wounds, but
without any enswathements. The rats of C group were put in a specially made Plexiglas box to receive whole-body irradiation with a $^{60}$Co gamma ray source. The dosage was 6 Gy, which had been confirmed to significantly delay the process of wound healing. The rats of this group were then anesthetized, and the same linear incisions were made as in the S group within 30 min after irradiation. All the animals were separately caged with general food and drink.

On the 3rd and 5th days of posttrauma, 5 rats of every group were killed. The skin around the wounds were sterilized with 75% ethanol; crumbs of the wound tissue were removed, pruned to 2 × 2 mm$^2$, and cultivated in IMDM culture medium with 10% (v/v) FBS, 100 U·ml$^{-1}$ penicillin, and 100 µg·ml$^{-1}$ streptomycin. About seven days later, fibroblasts isolated from the wounds were collected to observe their abilities associated with tissue repair.

The fibroblasts (primary passage) were digested with 0.25% trypsin by a standard technique for adherent cells. The cell concentration was then adjusted to 2 × 10$^4$ fibroblasts and fibroblasts were cultured in 50 ml culturing bottles, each one containing 4 × 10$^4$ fibroblasts. Three days later, the fibroblasts were digested again, and the quantity was counted to evaluate their growth. Furthermore, the contents of proliferation cell nuclear antigen (PCNA) in fibroblasts were determined by SABC immunohistochemistry method to evaluate the proliferation of fibroblasts. Specimens were stained for PCNA at 1:100 dilution. The results of immunohistochemistry were semiquantified by an image-analysis software package, and the values of average optical density (ODu) × area (mm$^2$) were used to represent the contents of PCNA in fibroblasts.

Fibroblasts (primary passage) were digested, and they were recultivated in 50 ml culturing bottles, each bottle containing 4 × 10$^4$ fibroblasts. Six hours later, the unattached fibroblasts were collected and the quantity was counted. The abilities of attachment (AT) were calculated with this equation:

$$AT(\%) = \frac{\text{total cell numbers} - \text{unattached cell numbers}}{\text{total cell numbers}} \times 100\%.$$

Fibroblasts (passage one) were digested with 0.25% trypsin for 10 min; the digestion was then stopped by the addition of serum. Free fibroblasts were collected and the quantity was counted. After that, adherent fibroblasts were collected by a special cell scraper, and the quantity was counted. The following equation was used to calculate the abilities of the adhesion (AD) of fibroblasts in wounds:

$$AD(\%) = \frac{\text{adherent cell numbers}}{\text{free cell numbers} + \text{adherent cell numbers}} \times 100\%.$$

Apoptosis is a kind of cellular suicidal behavior, which plays very important roles in both physiological and pathological states. In the present study, terminal uridine nick-end labeling (TUNEL) assay was used to detect apoptosis of fibroblasts in wounds. Cells with TUNEL positivity were quantified under a ×40 objective, and five randomly selected fields of each section were observed. The apoptotic rate = TUNEL-positive cells/total cells.

All data were imported into a computer and analyzed with an SPSS software pack. One-way ANOVA was used in the statistical analysis. The results were expressed as means ± SEM, and a value of $p < 0.05$ was accepted as significant.

**RESULTS AND DISCUSSION**

The period from the 3rd to the 5th day is the early phase of tissue repair, and it is the stage for the initiation of granulation tissue formation and wound healing, so it is an important and crucial period for wound healing. Fibroblasts play critical roles in the processes of wound repopulation and subsequent tissue remodeling. The proliferation of fibroblasts in wounds is an important index to represent the abilities associated with tissue repair. PCNA has been identified in quaternary complexes with the cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitor P21. PCNA can also bind to many proteins, particularly to the replication protein (such as DNA ligase I) as a target for CDKs. Furthermore, PCNA can stimulate the phosphorylation of DNA ligase I; thus PCNA plays important roles in the processes of DNA replication and the regulation of cell cycles, and it is considered an index of proliferation. We found in the present study that the contents of PCNA in fibroblasts from wounds combined with WBI were significantly decreased in comparison with those of simple incision (Fig. 1). Moreover, the results of direct fibroblast

**Fig. 1.** Immunohistochemical staining for PCNA in fibroblasts in wounds (SABC). The brown stainings are the nucleus of PCNA positive fibroblasts because the PCNA is in the nucleus of positive cells (arrow). Scale bar, 25 µm. A: Immunohistochemical staining for PCNA in fibroblasts in wounds of the S group. B: Immunohistochemical staining for PCNA in fibroblasts in wounds of the C group.
Activity of Fibroblasts in Connection with Tissue Recovery in Wounds


Table 1. The changes of the abilities associated with the tissue repair of fibroblasts in wounds.

<table>
<thead>
<tr>
<th></th>
<th>S group</th>
<th>C group</th>
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<tbody>
<tr>
<td></td>
<td>3rd day</td>
<td>5th day</td>
</tr>
<tr>
<td>Number (1×10⁴/ml)</td>
<td>19.50 ± 1.56</td>
<td>13.21 ± 1.15</td>
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<tr>
<td>PCNA (ODu × mm²)</td>
<td>386.35 ± 35.15</td>
<td>269.29 ± 29.14</td>
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<tr>
<td>Attachment (%)</td>
<td>90.56 ± 7.25</td>
<td>88.28 ± 7.69</td>
</tr>
<tr>
<td>Adhesion (%)</td>
<td>49.55 ± 4.23</td>
<td>34.59 ± 3.87</td>
</tr>
<tr>
<td>Apoptotic ratio (%)</td>
<td>12.08 ± 1.68</td>
<td>18.71 ± 1.57</td>
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n = 5, mean ± SD. *p < 0.05, compared with the S group.

Fig. 2. Apoptosis of fibroblasts in wounds (TUNEL). The apoptotic cells detected by TUNEL assay are also the brown stained in the nucleus. So the brown-stained cells are the apoptotic fibroblasts. Scale bar, 25 µm. solid arrows, TUNEL positive (apoptotic) fibroblasts; open arrows, TUNEL negative (unapoptotic) fibroblasts. A: Apoptosis results of fibroblasts in wounds of the S group. B: Apoptosis results of fibroblasts in wounds of the C group.

count showed that the quantity of fibroblasts from wounds combined with WBI was significantly less than those from simple incisions (Table 1). These data suggested that the proliferation of fibroblasts from wounds combined with WBI were significantly weaker than those of simple incisions. The amount of the fibroblasts can be considered as cell death in connection with the radiation, which may be expressed as apoptosis, a specific program to the radiation death of cells. The TUNEL results showed that the apoptotic ratio of fibroblasts from wounds combined with WBI increased significantly in comparison with that of simple incisions (Fig. 2, Table 1), which might also result in the decrement of fibroblasts and granulation tissues. Therefore the apoptosis increment of fibroblasts also suggested that the abilities associated with the tissue repair of fibroblasts in wounds combined with WBI were significantly decreased. The abilities of the attachment and adhesion of fibroblasts in wounds are also associated with tissue repair, which are the similar index as viability. The present results showed that the abilities of attachment and the adhesion of fibroblasts from wounds combined with WBI significantly decreased in comparison with those of simple incision (Table 1). These data may suggest that the activity of fibroblasts in connection with tissue recovery was significantly impaired in the wounds with WBI.

Thus the present results, that the activity of fibroblasts in connection with tissue recovery was significantly impaired in wounds with WBI, suggested that WBI had damaging effects on fibroblasts in wounds. Furthermore, our previous in vivo data showed that the amount of tissue-repairing cells, such as fibroblasts and macrophages, in wounds combined with WBI was significantly less than that of simple incision. Moreover, we found that ⁶⁰Co gamma-ray had damaging effects on cultured fibroblasts and that irradiation-injured fibroblasts were less responsive to the stimulation of cytokines. These data suggested that WBI exerted damaging effects on tissue-repairing cells, which might be one of the primary reasons why the healing of wounds combined with WBI was significantly delayed.

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