

Transient infiltration of neutrophils into the thymus in association with apoptosis induced by whole-body X-irradiation

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Abstract: Generally, the process of apoptosis does not cause leakage of noxious cytosolic contents and is therefore non-inflammatory. However, as previously shown, macrophages ingesting apoptotic CTLL-2 cells produced pro-inflammatory cytokines, particularly interleukin-8 (IL-8) and macrophage inflammatory protein-2 (MIP-2), a murine IL-8 homolog. This predicted that rapid and massive apoptosis may induce neutrophil accumulation *in vivo*. In this study, we tested this prediction by inducing apoptosis by whole-body X-irradiation in mice. After exposure to 4 Gy X-ray irradiation, mice exhibited considerable apoptosis of thymic cells, which was associated with transient infiltration of neutrophils as well as MIP-2 mRNA expression. In contrast, in p53-deficient mice in which irradiation-induced apoptosis was suppressed, as has been reported, infiltration of neutrophils into the thymus was less than that found in p53^{+/+} mice. Taken together, these results suggest that massive and rapid apoptosis can result in infiltration of neutrophils. *J. Leukoc. Biol.* 67: 780–784; 2000.

Key Words: cytokines · FACS staining

INTRODUCTION

In physiological and pathological conditions, professional phagocytes (macrophages) are largely responsible for the clearance of apoptotic cells [1, 2]. Generally, the process of apoptosis does not cause leakage of noxious cytosolic contents and is therefore non-inflammatory [3]. We therefore tested the hypothesis that the interaction of phagocytes with apoptotic cells indeed fails to induce any pro-inflammatory signals [4, 5]. To our surprise, as previously shown, macrophages ingesting apoptotic CTLL-2 cells produced pro-inflammatory cytokines, in particular, the human CXC chemokine interleukin-8 (IL-8) [5] and macrophage inflammatory protein-2 (MIP-2) [4], a murine IL-8 homolog. In addition, the supernatant of the coculture of macrophages with apoptotic cells induced neutrophil infiltration after injection into the peritoneal cavity [4]. In general, during activation with inflammatory stimuli, resident macrophages partially induce local tissue and vascular changes, which cause migration of neutrophils and monocytes and ingestion of damaged tissue. We therefore predicted that rapid and massive apoptosis may induce neutrophil accumulation *in*

vivo, ultimately causing inflammation. In this study, we tested this prediction by giving whole-body irradiation to mice because whole-body X-irradiation is well known to cause rapid and massive apoptosis in the lymphoid organs such as the thymus [6].

MATERIALS AND METHODS

Preparation of cells

Female B10.Thy 1.1 mice were bred from the colonies at the Animal Production Facility of the National Institute of Radiological Sciences and were maintained within a microbiologically clean conventional animal facility [7]. These mice (8 weeks old) were used throughout, except in the experiment shown in Figure 4. In that experiment, p53-deficient and wild-type mice (C57BL/HeN) were used. Whole-body X-irradiation was performed with a Shinai-III X-ray generator (Shimazu Seisakusho Ltd., Kyoto, Japan) at 0.57 Gy/min (Irradiation Parameters, 200 KVp, 20 mA, with filters of 0.5-mm Cu and 0.5-mm Al; half-value layer, 1.24-mm Cu; target-skin distance 52 cm; 1 Gy = 100 rad), and apoptosis was induced with X-rays (4 Gy). The thymus was removed from cervical dislocated mice at the indicated time, and cells were teased out with pincettes. Thymuses were suspended in 5 mL RPMI 1640 medium, and the total cell number was counted. The cell viability was assessed with trypan blue dye exclusion.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cells of the thymus, and RT-PCR was performed as previously described [4]. The primers (5' primer and 3' primer), annealing temperatures, concentrations of MgCl₂, and predicted sizes for PCR products were as follows. MIP-2: GAAGTGGCTGTCAATGCCT, GTTAGCCTTGCCCTTGTGCA, 61°C, 2 mM, 204 bp. β_2 -microglobulin: TCTGGTGCTTGTCTACTGA, GAAAGACCAGTCTTGCTGA, 61°C, 2 mM, 220 bp [4]. The PCR products were digested with appropriate restriction enzyme(s) to confirm specificity.

Flow cytometric analysis

Fifty microliters of each cell suspension at a cell density of 10⁷ cells/mL was incubated with 1 μ g of fluorescein isothiocyanate (FITC)-labeled anti-Gr-1 mAb (mouse neutrophil marker [8]), anti-Mac-3 mAb (mouse macrophage marker [9]), or isotype control rat IgG1 κ in an individual tube for 30 min at 4°C (all antibodies were purchased from PharMingen, San Diego, CA). The cells were then washed twice with phosphate-buffered saline (PBS), followed by

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analysis with FACScan and CellQuest software (Becton Dickinson, Mountain View, CA) as previously described [10]. The exposure of phosphatidylserine (PS) was estimated by staining cells at a density of 10^7 cells/mL with FITC-annexin V under the conditions recommended by the manufacturer (Bender MedSystems, Vienna, Austria) [5].

RESULTS AND DISCUSSION

A moderate dose of whole-body X-irradiation reportedly causes rapid and massive apoptosis in the lymphoid organs such as the thymus [6], and apoptotic cells are phagocytosed by macrophages [11]. The apoptosis induced by whole-body X-irradiation was thus chosen as a model to test our prediction that massive apoptosis occurring rapidly may induce neutrophil accumulation *in vivo*.

PS externalization, one of the well-known markers of apoptosis, reached a peak at 6 h after irradiation, followed by loss of membrane permeability, which reached a peak at 12 h after irradiation (Fig. 1B). Thymocytes exhibited a decrease in cell size, another well-known marker of apoptosis, at 3 h after irradiation (data not shown). The percentage of cells expressing PS on the cell surface returned close to control levels by 24 h

after irradiation, which is consistent with the report that apoptotic cells are phagocytosed by macrophages in the thymus [11]. We also observed phagocytosis of apoptotic cells by macrophage-like cells histochemically (Fig. 2A, indicated with asterisks) where some macrophage-like flat cells contained apoptotic body-like particles. After irradiation, the total cell number in the thymus, including dead cells, decreased in a time-dependent manner to approximately 10–20% of the control at 24 h (Fig. 1C), which is generally believed to reflect phagocytosis of apoptotic cells. The viability of thymocytes was reduced to approximately 50% by 12 h after irradiation.

Flow cytometric analysis with FITC-anti Mac-3 antibody demonstrated that the total number of macrophages initially increased approximately threefold by 3 h after irradiation but decreased thereafter to the control level at 12 h (Fig. 1D). Horino et al. reported that the monocyte chemotactic factor, S19 ribosomal protein dimer, was released by apoptotic cells and facilitated the phagocytic clearance of apoptotic cells [12]. However, this mediator might not be responsible for the macrophage accumulation described here because the number of macrophages rapidly increased before the onset of apoptosis. Although it is not known at present what accounts for the rapid

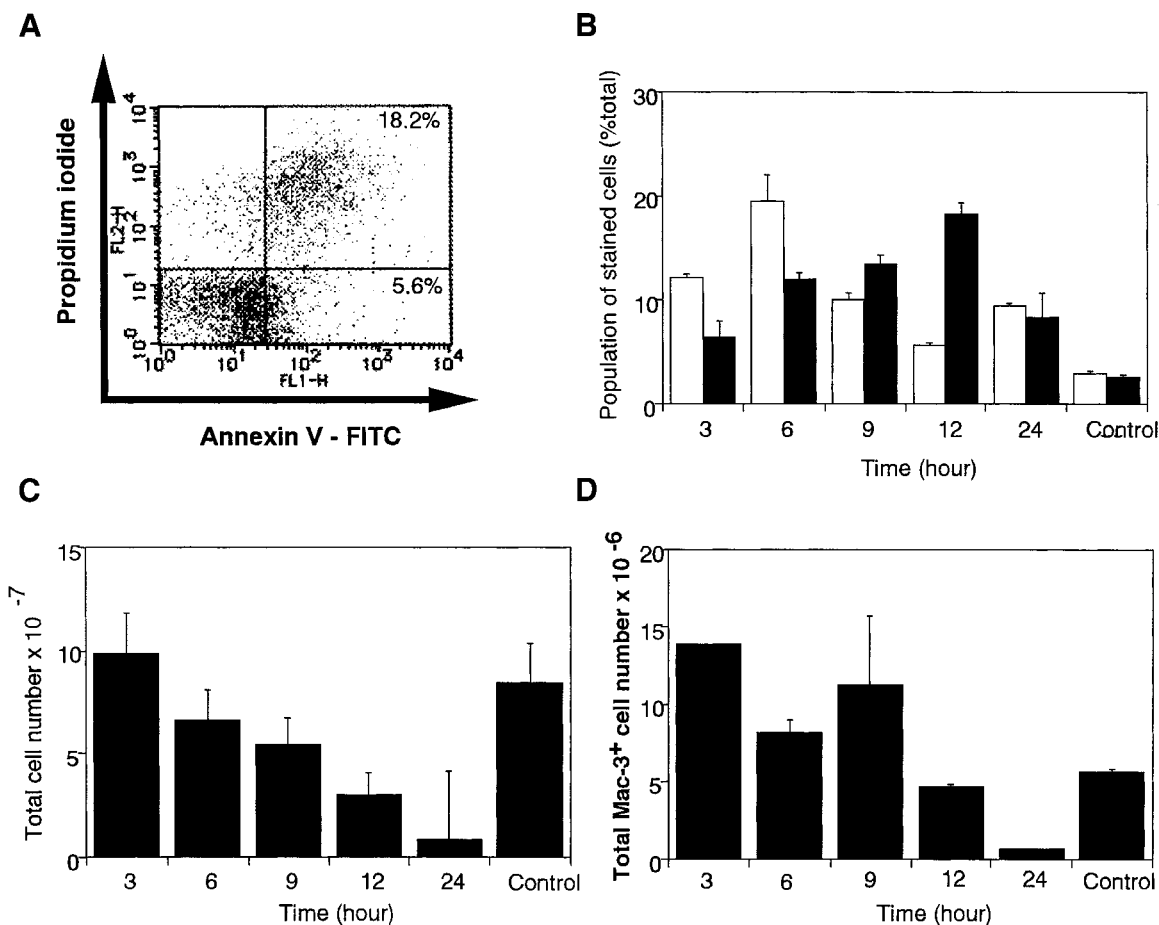


Fig. 1. Kinetics of changes in cell population after whole-body irradiation. Mice were irradiated with X rays (4 Gy), followed by analysis of cell-surface markers of thymocytes with a flow cytometer at indicated times. (A) Flow cytometric analysis of cells at 12 h after irradiation that were stained with FITC-annexin V and propidium iodide. In this case, the percentages of cells stained with FITC-annexin V (early apoptotic cells, lower right) and cells stained with propidium iodide and FITC-annexin V (late apoptotic cells, upper right) were 5.6 and 18.2%, respectively. (B) The percentages of early apoptotic cells (open bars) and late apoptotic cells (filled bars). (C) Total cell number of the thymus. (D) Total macrophage (defined as Mac-3-positive cells) number of the thymus. Data are expressed as means \pm SE of two to five mice in panels B, C, and D.

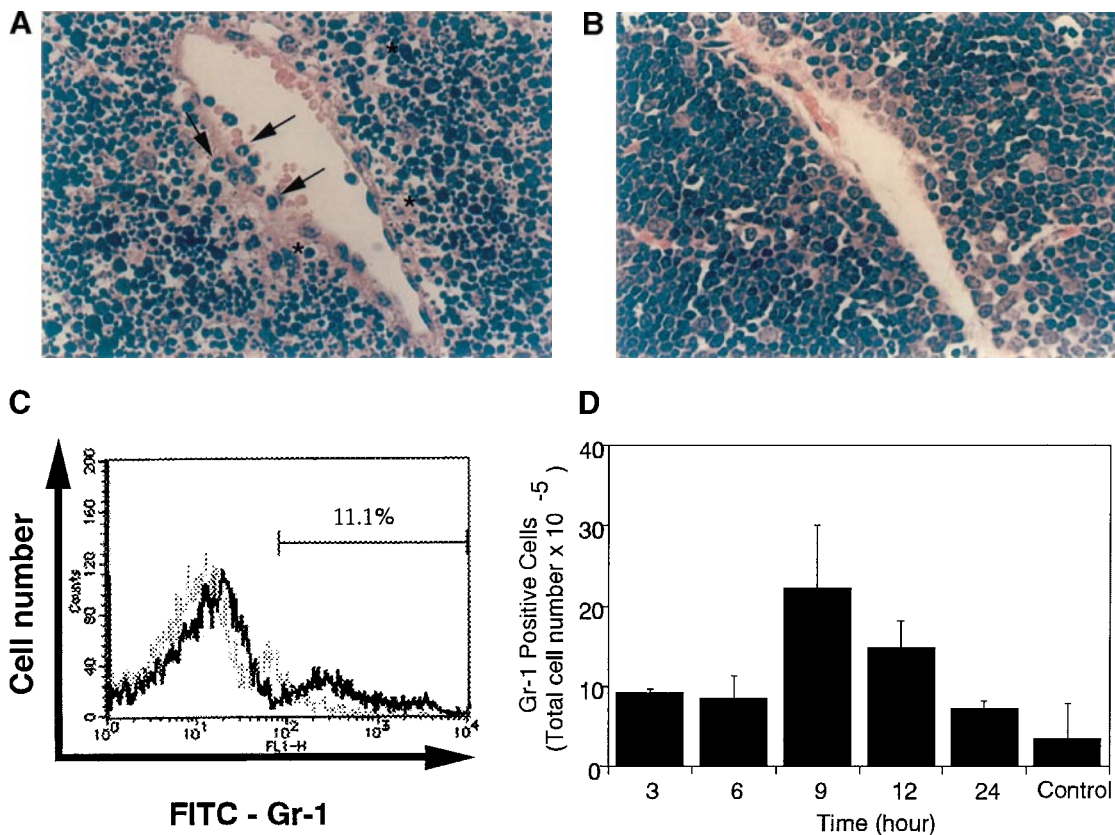


Fig. 2. Neutrophil infiltration in thymus after whole-body X-ray irradiation and its kinetics. (A, B) Mice were irradiated with X-rays (4 Gy), followed by hematoxylin and eosin staining of sections of the thymus at 9 h (A, original magnification $\times 400$) and the control thymus (B, original magnification $\times 400$). The thymus was fixed with formalin, embedded in paraffin, and sectioned. (C) Analysis of cell surface marker (Gr-1) of neutrophils at 12 h after irradiation with a flow cytometer. Bold line indicates an experimental profile with anti-Gr-1 mAb; dotted line indicates a control profile with control Ab. In this case Gr-1-positive cells were 11.1%. (D) Analysis of cell-surface marker (Gr-1) of neutrophils with a flow cytometer at indicated times. Data are expressed as means \pm SE of three mice. Values at 9 and 12 h after irradiation were significantly ($P < 0.01$ and $P < 0.05$, respectively; paired *t*-test) different from control.

macrophage accumulation, it is possible that irradiation may induce the production of macrophage chemotactic factor(s) before initiation of apoptosis.

Neutrophils also appeared in the thymus of irradiated mice (Fig. 2A, indicated by arrows), but the total number of neutrophils peaked at 9 h, and persisted longer with a relatively large number of neutrophils even at 12 h (Fig. 2D). RT-PCR analysis detected mRNA of MIP-2, one of the CXC chemokines and a murine IL-8 homolog, in the irradiated thymus at 9 h and faintly at 6 and 12 h (Fig. 3), suggesting the possibility that MIP-2 may be responsible for the neutrophil accumulation. At present we cannot exclude the possibility that factors other than MIP-2 are also responsible for neutrophil accumulation. In this

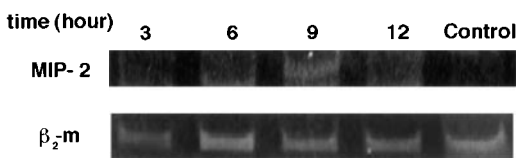


Fig. 3. MIP-2 mRNA expression in the thymus after whole-body X-ray irradiation. Mice were irradiated with X-rays (4 Gy), followed by analysis of MIP-2 and β_2 -microglobulin mRNAs of thymocytes at indicated times. Polyacrylamide gel electrophoretic patterns of each RT-PCR product are shown.

study, we have not identified the cellular source of MIP-2 as macrophages, and therefore the possibility remains that apoptotic cells themselves released this neutrophil chemotactic factor.

To confirm the causal relationship between apoptosis and neutrophil accumulation, we employed p53-deficient mice, which were resistant to radiation-induced apoptosis [13]. As shown in **Figure 4A**, 4-Gy-irradiated p53-deficient mice showed a smaller percentage of apoptotic cells expressing PS on the cell surface than control mice. Neutrophil accumulation, when expressed as a percentage of Gr-1-positive cells, was also suppressed in p53-deficient mice compared with control mice (Fig. 4B). This indicated that apoptosis is closely associated with neutrophil accumulation. The total number of neutrophils, however, was not suppressed in p53-deficient mice, because the total cell number in the thymus of p53-deficient mice was two- to threefold greater than that of control mice after irradiation.

Myeloperoxidase immunostaining revealed the transmigration of neutrophils into the parenchyma as well as phagocytosis of neutrophils by macrophages (data not shown). In addition, some of the accumulated neutrophils were stained with FITC-annexin V and propidium iodide, whereas the others were not

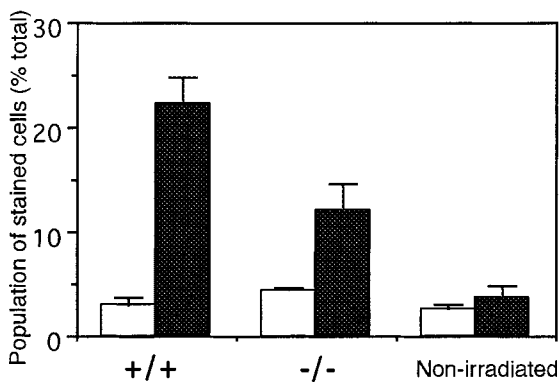
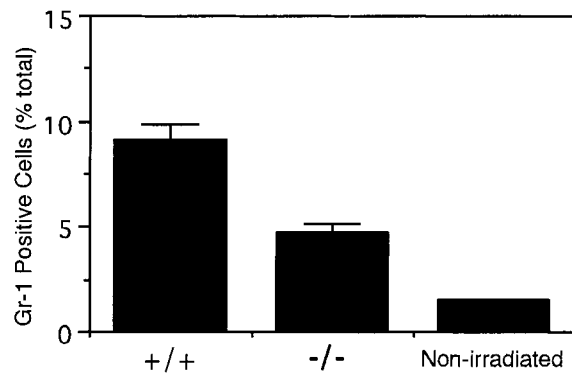
A**B**

Fig. 4. Apoptosis and neutrophil infiltration in the thymi of wild-type and p53-deficient mice (C57BL/HeN). Wild-type and p53-deficient mice were irradiated with X-rays (4 Gy), followed by analysis of apoptosis and cell surface marker (Gr-1) of neutrophils at 12 h after irradiation. (A) The percentages of cells stained with FITC-annexin V (early apoptotic cells, open bars) and cells stained with propidium iodide and FITC-annexin V (late apoptotic cells, filled bars). (B) Percentage of Gr-1-positive cells. Data are expressed as the means \pm SE of two to three mice.

(data not shown). It is likely, therefore, that such viable neutrophils may play some role in the thymus. There are several possibilities. The first possibility is that neutrophils contribute to the clearance of apoptotic cells, in particular late apoptotic cells with loss of membrane permeability, which have not been taken up by macrophages. The second possibility is that neutrophils assist in the reconstruction of the thymus by producing as yet unidentified cytokines. The third possible consequence of accumulated neutrophils is to cause severe damage to tissues. Although we favor the first possibility, we cannot exclude other possibilities at present, and certainly they are worthy of further study.

In endotoxin-induced liver failure, tumor necrosis factor α caused apoptosis of the hepatic parenchymal cells, which led to neutrophil infiltration, although neutrophil chemotactic factor and its cell source were not identified [14]. Although the authors believed hepatic parenchymal cells to be the producer of chemotactic factor(s), it is also possible that macrophages phagocytosing these apoptotic cells are the cell source. Once chemotactic factor(s) is produced in response to apoptosis, neutrophils accumulate and thus contribute to the pathogenesis of endotoxin-induced liver failure.

In multicellular organisms, neither normal morphogenesis nor normal cell turnover is associated with inflammation [15, 16]. In such physiological processes, apoptotic cells are believed to be phagocytosed as soon as they appear because apoptotic cells are rarely detected in normal tissue [11]. Our previous study as well as other studies indicated that early apoptotic cells were less potent than late apoptotic cells in the induction of IL-8 after phagocytosis [5, 17]. This may be one of the conditions in which the production of proinflammatory cytokines by macrophages after phagocytosis of apoptotic cells does not occur. The second possibility is quantitative in that apoptosis occurs so sporadically *in vivo* [18] so that little or no proinflammatory cytokines are produced. The third possibility is production of anti-inflammatory cytokines. During relatively long coculture of apoptotic cells with macrophages, i.e. 18 h, transforming growth factor β , prostaglandin E_2 , and platelet-activating factor were reportedly produced to suppress inflam-

matory cytokine production [19]. However, extensive apoptosis as in liver failure or as a result of irradiation damage are clearly associated with the recruitment of acute inflammatory neutrophil infiltration.

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