Enhancement of Radiation-induced Apoptosis by Preirradiation with Low-dose X-rays in Human Leukemia MOLT-4 Cells

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Low Dose/Adaptive Responses/Apoptosis/MOLT-4/p53.

The effects of low-dose preirradiation on the process of radiation-induced cell death were investigated in human leukemic MOLT-4 cells. By 0.2 Gy of X-rays given 12 h prior to a challenge dose of 5 Gy, the process of apoptosis was accelerated. The acceleration was associated with a certain increase in caspase 3 activity, a disruption of the mitochondrial transmembrane potential, and an accumulation of p53 proteins. This finding is in contrast to the radiation adaptive responses in which a small dose of preirradiation would induce certain radiation resistance and decrease the cell death after irradiation with higher doses.

INTRODUCTION

A small dose of radiation is known to induce a radioresistance against larger doses given later. Since the first discovery by Olivieri et al.,1 this phenomenon, the radiation adaptive response, has been observed in a variety of cells and organisms.2,3 We also reported that a “conditioning dose” of 0.2 Gy slowed down the cell death process in human fibroblasts exposed to a challenge dose of 3 Gy.4 The adaptive response was first reported in human lymphocytes in terms of chromosome aberrations; however, when apoptosis was used as an end point, an enhancement of cell death was reported.5 We also previously found that small doses of X-rays accelerated the process of cell death, using a dye exclusion test in MOLT-4 cells of human T-cell leukemia origin, in which radiation-induced cell death has been well characterized as apoptosis.6–8

A dye exclusion test with erythrosine or trypan blue detects a disruption of membrane permeability, one of the final end points of apoptosis. Before the disruption of permeability there are some structural changes in cellular membrane. One of these changes is the appearance of phosphatidylserine on the surface of the cell membrane, which can be detected with annexin V.

The membrane change is preceded by the activation of caspases, the central mediators of programmed cell death. They are cysteine proteases, which cleave their substrates at specific aspartate residues. Caspase-3 is an effector caspase that once activated by the initiator caspases cleaves diverse cellular substrates, including poly ADP-ribose polymerase, caspase-dependent DNase inhibitor, and others,9,10 to execute the cell death program. There is a group of caspases called initiator caspases, such as caspase 8 and caspase 9, that activates the effector caspases. Caspase 9 is activated by a factor called apaf-1, containing cytochrome c, that is released from mitochondria as a cofactor. Mitochondria have been discovered to exert an important, perhaps essential role in the process of apoptosis.11,12 An increase in the permeability of the inner and/or outer mitochondria membrane appears to be accompanied by the release of proteins normally confined to the intermembrane space.13–15 Cytochrome c is an electron-transporting protein that resides within the intermembrane space of the mitochondria where it plays a critical role in the process of oxidative phosphorylation and the production of cellular ATP. Thus the collapse of membrane potential is a good indicator for the change in mitochondria.

The mitochondria change is a consequence of the interaction with Bcl family proteins, including Bax. The transcription of Bax is regulated by p53. A key player in cellular radiation responses, it is involved in a cell cycle checkpoint in G1 and G2/M transition, in DNA repair, and in apoptosis.16–23 p53 is a DNA-binding tumor suppressor that contains an oligomerization and transcription activation domain and upregulates growth arrest and apoptosis-related genes in response to stress signals, thereby influencing programmed cell death, cell differentiation, and the cell cycle control mechanism.24

Because by use of a dye exclusion test we observed an enhancement or acceleration of radiation-induced cell death, we aimed in the present study to elucidate the mechanism of
the acceleration, to find which step in the apoptotic process is accelerated. We investigated some of the events in the upper stream of the apoptotic process: appearance of phosphatidyl serine as revealed by Annexin V staining, change in the activity of one of the caspases, change in mitochondrial, and stabilization of p53.

**MATERIALS AND METHODS**

**Cell culture**

Human leukemia MOLT-4 cells were cultured in RPMI 1640 medium (GIBCO/BRL) supplemented with 10% heat-inactivated fetal calf serum at 37°C in a humidified incubator with 5% CO2-air.

**Irradiation**

X-irradiation was performed with an X-ray generator (0.2 mm Cu and 1 mm Al filter, 150 kV, 5 mA, Hitachi). The irradiation was carried out at room temperature at a dose rate of 0.2 Gy/min.

**Detection of apoptosis**

An Annexin V-FITC kit (Beckman Coulter, Inc.) was used. Cells incubated for the indicated time after X-irradiation were collected by centrifugation at 400 × g for 3 min at 4°C. The cell pellet was resuspended in 0.5 ml ice-cold binding buffer. Five µl of annexin V-FITC solution and 5 µl of propidium iodide (PI) were added into the cell suspension and kept at ice-cold temperature for 10 min in the dark. Apoptotic cells were analyzed by using an EPICS XL flow cytometry system (Beckman Coulter, Inc.) with the EPICS EXPO32 software. More than 5,000 cells were subjected to the analysis.

**Caspase–3 activity assay**

To measure the activity of caspase 3, PhiPhiLux kit (Bio Vision) was used. The 10^6 cells pellet was suspended in 60 µl of substrate solution. After incubation at 37°C for 1 h, 1 ml of dilution buffer for the flow cytometer added. After centrifugation for 3 min at 1,000 × g, the pellet was loosened with 1 ml dilution buffer, and a sample was assayed within 30 min with the EPICS flow cytometer.

**Measurements of mitochondria membrane potential**

Five µl of 4 µM DiOC6 (3) were added to a 1 ml cell suspension to a final concentration of 20 nM, which was then kept for 30 min. The cells were resuspended in PBS (–). A cytofluorometric assay was performed within 10 min with the flow cytometer for a measurement of mitochondrial membrane potential (ΔΨm).

**Western blotting analysis of p53 protein**

The cells were suspended in 50 µl of chilled cell lysis buffer (Bio Vision) and kept on ice for 10 min. The supernatant was collected, and 15 µg of proteins were loaded onto a 12.5% SDS-PAGE for electrophoresis and transferred to a poly vinilidene difluoride membrane in 5% nonfat milk in phosphate-buffered saline. Immune blotting was performed with mouse monoclonal primary antibody p53 Ab-3 (Neo-markers, USA). The blots were further treated with horseradish peroxide-linked second antibody and specific bands were visualized by chemiluminescence (ECL, Amersham). The bands of p53 were scanned with a Chemi Doc system (Bio-Rad); the density of the signal was normalized with that of G3PDH.

**RESULTS**

**Dose-dependence of X-ray-induced apoptosis**

At an earlier stage of apoptosis, the cells are stained only by annexinV and later also by PI. Figure 1 shows the dose response of the cell population at various stage of apoptosis:

![Fig. 1. Dose-dependent induction of apoptosis 12 h after exposure to X-irradiation in MOLT-4 as measured by staining with FITC-Annexin V and PI. (A) Apoptosis induced 12 h after irradiation was plotted as a function of radiation dose. (B) The low dose region (0–0.1 Gy) in panel A was expanded. Open squares, closed triangles, and closed circles represent cells stained with annexin V (Ann [+]), cells stained with annexinV but not with PI (Ann [+] PI [–]), and cells stained with PI (PI [+]), respectively.](image-url)
one stained with PI [PI (+)], one stained with annexin V but not with PI [Ann (+) PI (–)], and the other with annexin V [Ann (+)]. The Ann (+) fraction includes both the Ann (+) PI (–) fraction and the fraction stained with both annexin V and PI. Because the Ann (+) fraction showed the most sensitive linear dose response curve, we chose this fraction as an end point of apoptosis in this study. The low-dose region (< 1 Gy) is expanded in Fig. 1B. A linear dose-dependent response can be seen to exist in the range of 0.2–1.0 Gy; however, only a little increase, if any, was detected for doses below 0.2 Gy.

Effect of preirradiation on the time course of apoptosis

Figure 2 shows the time course of apoptosis after 5 Gy irradiation with or without 0.2 Gy preirradiation. In cells exposed to 5 Gy alone, the significant increase in apoptotic fraction was not observed 4 h after the irradiation; in those cells exposed to 0.2 Gy 12 h before 5 Gy, however, the significant increase in apoptosis was observed at the same timing (time?). Therefore the fractions of apoptosis were always larger in preirradiated cells.

Caspase 3 activity and mitochondria membrane potential change accompanied by apoptosis

To investigate whether earlier steps in the process of apoptosis are accelerated, we examined the activity of caspases and the change in the mitochondrial membrane. Figure 3 shows caspase 3 activity (panel B) and membrane potential of the cells (panel C), along with the apoptosis (panel A) in MOLT-4 cells.

The activity of caspase 3 was enhanced by 5 Gy alone. Preirradiation of the cells with 0.2 Gy further increased the activity (Fig. 3B). The activities of other caspases of the caspase family, i.e., caspase 2, 3, 6, 8, and 9, were also enhanced in the same manner as caspase 3 (data not shown).

Accumulation of p53 after irradiation

To further pursue the mechanism of the acceleration of apoptosis enhancement, we investigated the effect of low-dose irradiation on the accumulation of p53 proteins; p53 is a key protein to control apoptosis. Figure 4 shows Western blots and the p53 protein level in 5 Gy-irradiated MOLT-4 cells with or without 0.2 Gy preirradiation. Because preliminary experiments revealed that a peak of p53 protein accumulation after 5 Gy alone was reached at 4 h after irradiation (data not shown), we chose this timing for the p53 analysis. The accumulation of p53 proteins was enhanced by the 0.2 Gy preirradiation in parallel with the enhancement of apoptosis.
We previously reported the acceleration of cell death process in MOLT-4 cells in terms of stainability with erythrosine, an end point at the very end of the apoptotic process. To elucidate the mechanism underlying the acceleration, we investigated the earlier steps of apoptosis: the cellular membrane integrity (Figs. 1, 2); caspase-3 activity (Fig. 3B); mitochondrial potential (Fig. 3C); and p53 accumulation (Fig. 4). Each of these end points showed a parallel correlation with apoptosis when the challenge dose of 5 Gy was combined with the conditioning dose of 0.2 Gy. It should be noted, however, that a significant difference after the conditioning dose of 0.2 Gy alone was observed only for p53 accumulation (Fig. 4), but not for the change in cell membrane structure (Fig. 3A), caspase 3 activity (Fig. 3B), or mitochondrial change (Fig. 3C).

These findings may explain the acceleration of the apoptotic process by the conditioning dose. If there is a kind of threshold for the amount of p53 to activate the expression of certain proteins to trigger the process of apoptosis, and if the amount of p53 accumulated after 0.2 Gy is below the threshold, the amount of p53 could reach the threshold within a shorter period after the challenge dose, resulting in the acceleration of apoptosis as shown in Fig. 2.

Contrary to the acceleration of apoptosis shown in the present study, in some types of cells, such as cultured malignant cells and mouse spleen cells, the suppression of apoptosis was reported. As a possible mechanism, the attenuation of p53 response has been postulated. In cellular responses to ionizing radiation, p53 plays very important roles. It regulates DNA repair, which would lead cells to survive, and also apoptosis, which leads cells to die. If the level of threshold for triggering the two processes is different in different types of cells, the different responses depending on cell type could then be explained.

There has been an argument on the involvement of the p53 pathway. Nakano et al. claimed the involvement of p53 by demonstrating that the transfection of mutant-type p53 delayed the process of apoptosis. Kuwabara et al. emphasized the involvement of p38/JNK pathway in apoptosis, but the two pathways are not necessarily mutually exclusive. The results shown in the present study suggest the at least partial involvement of p53 in the regulation of apoptosis.

The significance of the adaptive response in radiation protection has been discussed; it represent the increase in the protective system against damage induced by high doses of radiation. The enhancement of apoptosis shown in the present study would represent another protective response against genetic damage because it would remove potentially malignant cells that contain a certain type and amount of DNA damage.

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