Suppression of p53 and Bax Accumulation after X-irradiation by Small Dose Preirradiation in Radioadaptive Survival Response of C57BL/6 Mice

Morio Yonezawa¹, Akihisa Takahashi², Ken Ohnishi², Jun Misonoh³ and Takeo Ohnishi².

¹Research Institute for Advanced Science and Technology, Osaka Prefecture University, Japan
²Department of Biology, Nara Medical University, Kashihara, Japan
³Research Institute of Electric Power Industry, Abiko, Japan.

INTRODUCTION

It has been reported by the authors that X-irradiation with 0.30-0.50 Gy two weeks before a second exposure to a mid-lethal dose significantly reduced the bone marrow death rate in ICR and C57BL/6 strains of mice (1-3). Though radioadaptive response within blood-forming tissues has been speculated on the induction the radioresistance, molecular mechanism has been unknown yet. The present study was carried out to elucidate molecular mechanisms for the radioadaptive survival response. Effect of preirradiation (0.45 Gy) on p53 as well as Bax accumulation after challenging irradiation was studied in C57BL/6N mice.

MATERIALS AND METHODS

Animals

SPF 4-week-old male mice of C57BL/6N strain, purchased from Charles River Japan Inc. were used. They were kept in a clean-conventional environment, and given acidified water, adjusted to about 2.7 by adding hydrochloric acid into tap water to prevent contamination with *Pseudomonas* bacteria. The study was conducted according to the Guideline for Animal Welfare and Experimentation issued by the Research Institute for Advanced Science and Technology, Osaka Prefecture University.

Irradiation

The animals were whole-body irradiated in a revolving, partitioned, plastic chamber with X-rays (260 kV, 10.5 mA, 0.3 mm Al + 0.5 mm Cu filter, HVL 0.90 mm Cu, 0.45 Gy/min). The priming dose was 0.45 Gy (or sham-treated for 1 min), and the challenging dose was given two weeks afterwards. For all experiments, sham-irradiated controls were run concurrently with the pre-irradiated groups.

Short term p53 accumulation in the tissues after irradiation determined by the Western blotting method

A key protein p53, which mediates signal transduction (9) either to transient G1 arrest (repairs DNA damages and increases cell survival) or to apoptosis (cell death), was measured by the Western blotting method to study molecular mechanism of the induction of the radioresistance. The assay was applied to 6 tissues (the brain, thymus, right lung, spleen, a part of liver and upper half of small intestine) of mice of 4 groups: 0.0 Gy & 0.0 Gy (intact, 3 animals), 0.45 Gy & 0.0 Gy (priming irradiation only, 3 animals), 0.0 Gy & 3.0 Gy (Challenging irradiation only, 3 animals) and 0.45 Gy & 3.0 Gy (priming and challenging irradiations, 4 animals). The animals were killed by cervical dislocation five hours after the second irradiation with 3.0 Gy. The tissues were removed, washed with physiological saline, wiped with filter paper and kept in Eppendorf tubes. Bone marrow cells were collected from right and left femora with 1 ml of physiological saline. The samples were immediately chilled in liquid nitrogen, and kept at dry ice temperature until analysis. The tissues were then pulverized by freeze-fracturing in liquid nitrogen three times, and suspended in RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% (v/v) NP-40, 1% (w/v) sodium deoxycholate and 0.05% (w/v) sodium dodecylsulfate, SDS). The insoluble cell debris was removed by centrifugation at 18,500 x g for 10 min at 0°C. The supernatants were collected and the protein concentration was quantified using protein assay reagent (Bio-Rad Laboratories, Hercules, CA) and an UV-160A spectrophotometer (Shimadzu Co., Kyoto, Japan). The total protein samples (20 μg each) were subjected to 10% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE), then transferred electrophoretically to and Immo-bilon™ PVDF membrane (Millipore Co. Bedford, MA). The membranes were incubated with an anti-p53 monoclonal anti-body (Pab 421, which detects both wild-type and mutant p53 in mammalian cells, Oncogene Science Inc., Uniondale), and treated with a horseradish peroxidase-conjugated anti-mouse IgG anti-body (Zymed Labs. Inc., San Francisco, CA). The sensitivity of the visualization of the bands was enhanced using the BLAST blotting amplification system (Dupont/Biotechnology System NEN Research Products, Boston, MA). For confirmation of p53 band position, we used p53-deficient mouse embryonal fibroblast cells, MT158-8 (provided by Dr. O. Niwa, Kyoto Univ., Kyoto, Japan) as a negative control and protein size markers (Amersham Life Science, Buckinghamshire, U.K.). The densities of the bands were measured using a personal image analyzer LA-555D (PIAS, Osaka, Japan). The p53 protein values with standard errors presented in this paper are expressed as ratios of the amount of the two proteins in the irradiated groups (pre-irradiated and non-pre-irradiated) to that in the non-irradiated group.
**Histological study for p53 and Bax accumulation of the spleen**

The effect of the pre-irradiation was further examined in histological study for p53 and Bax accumulation of the spleen. The spleen was sampled on days 3 and 7 after the challenging irradiation with 3 Gy, and was fixed in 10% neutralized formalin-buffer solution. p53 and Bax on formalin-fixed paraffin-embedded sections were stained by the avidin-biotin peroxidase complex method using HISTOFINE SAB-PO (R) kit (Nichirei Co., Tokyo, Japan). Antigenicity was unmasked by heating in an autoclave. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol at room temperature for 30 min. The sections were exposed for 10 min to an excess of blocking solution, and were treated with polyclonal anti-p53 antibody (CM1, Novocastra Lab. Ltd., Newcastle, UK) or anti-Bax antibody (Ab-1, Oncogene Research Products, Cambridge, MA) for 2 h at 37 °C. Polyclonal anti-p53 and Bax antibodies were diluted at 1:100 and 1:20, respectively. Successively, the sections were incubated with secondary antibody and avidin-biotin peroxidase complex, were stained with diaminobenzidine, and were counterstained with Harris' hematoxylin. Cells with nuclei stained obviously were regarded as positive (+), and cells stained faintly in cytoplasm and nuclei were regarded as false positive (+/-). The tissue slices of the spleen, sampled 1 week after the challenging irradiation, were stained with p53 and Bax antibodies.

**RESULTS**

The pre-irradiation decreased bone marrow death of the irradiated C57BL/6 mice

For survival study, fifty animals were used for each group. The challenging X-ray dose was 6.75 Gy. The 30-day survival rates for the pre-irradiated and sham-irradiated control groups were 78.0% (39/50) and 8.0% (4/50), respectively (Fig. 1). The difference between the survival rates was significant (p<0.01), showing that the mice acquired radioresistance.

Short term p53 accumulation in the spleen after irradiation was inhibited by the pre-irradiation

Pre-irradiation itself did not affect p53 accumulation at the time just before challenging irradiation. Challenging irradiation with 3 Gy resulted in a significant increase (about 1.5 times) in p53 accumulation, but the pre-irradiation with 0.45 Gy inhibited the accumulation, even to the lower level of the intact animal group (Fig. 2). The result seemed likely to suggest that the pre-irradiation reduced apoptosis of the spleen cells after the challenging irradiation. p53 accumulation was not significantly affected by the preirradiation after 3 Gy in other tissues, namely the brain, thymus, lung, small intestine and bone marrow.

The pre-irradiation decreased both p53 and Bax positive cells in the spleen of irradiated mice

The preirradiation (with 45 cGy) itself did not give any change in p53 and Bax level of spleen cells 14 days later. This indicates p53 as well as Bax induction by the priming irradiation did not continue for 2 weeks. Challenging exposure itself accumulated both p53 and Bax in the spleen. Pre-irradiation prevented induction of both p53 and Bax positive cells following challenging exposure (Fig. 3 and Fig. 4, respectively). Since Bax protein has been recognized to direct cell to apoptosis (9), these results seem to show that the pre-irradiation diminished signal transduction to apoptosis after the challenging irradiation. Significant effects of the pre-irradiation were not observed from measurements 3 days after the challenging irradiation.

**DISCUSSION**

Several experiments were carried out to elucidate the molecular mechanism of the radio-adaptive response in whole animals using C57BL/6 mice. Radio-adaptive survival response was firstly confirmed in this strain of mice. The phenomenon is thought to be unrelated to and unpredictable from the high dose exposure experiments.

From the results above mentioned, we suppose that the molecular and biological mechanisms for the induction of the radio-resistance may be as follows:

(i) Death by apoptosis of highly radiosensitive cells after the pre-irradiation stimulated proliferation of healthy, radio-resistant, replacement cells during the interval time of 2 weeks. This cell-replacement hypothesis coincides with the postulation of Kondo for radiation hormesis (10).
(ii) Inhibition of both p53 and Bax accumulation in the spleen cells after challenging exposure by the pre-irradiation might result in a decrease in apoptotic cell death which might have been caused through signal transduction of these proteins, thus leaving much more hematopoietic cells alive.

(iii) This situation might favor recovery of hematopoietic tissues from radiation-induced acute injury, resulting in stimulated recovery from hematopoietic injury, and finally resulted in a decrease in bone-marrow death rate.

REFERENCES


