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Variability of the Adaptive Response to Ionizing Radiation Depending on Normal or Neoplastic Properties of the Cells

Sang-Hee Park[1], Yeon Hee Lee[2], Seong-Yul Yoo[1], Chul-Koo Cho[1] and Yun-Sil Lee[1]

[1]Laboratory of Radiation Effect, Korea Cancer Center Hospital, Seoul 139-706
[2]Department of Biology, Seoul Women's University
College of Natural Science, Seoul 139-705

Since the beneficial effects induced by low-dose radiation (0.01 ~ 0.5 Gy) was usually certified in normal cells, we compared the induction of adaptive responses of different properties of the cells: normal mouse lymphocytes (NL); L929 cells established from mouse connective tissue; primary mouse keratinocytes (PK); *v-ras*^{Ha} trasnformed mouse keratinocytes (ras-PK); line 308 from mouse papilloma; EL-4 cells from mouse lymphoma, radiation-sensitive lymphoma cells, L5178Y-S; radiation-resistant lymphoma cells, L5178Y-R. The time interval for the induction of the adaptive response was 4 hrs and 7 hrs. The adaptive response was observed in NL, PK, L929, and L5178Y-R, but not in L5178Y-S, EL-4, ras-PK, and 308 as determined by cell survival. Apoptosis was also reduced by priming the exposure of low-dose radiation in NL, PK, L929, and L5178Y-R cells, when detected using DNA ladder or TUNEL assay. These results suggest that adaptive response by low dose preirradiation is induced in normal or primed cells and apoptosis pathway is involved in these adaptive responses.

Key Words: Adaptive response, Gamma-ray, Low dose, Apoptosis, Normal cells, Neoplastic cells

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INTRODUCTION

Radiation is known to cause DNA damage and cell death in proportion to its dose. However, when cells are exposed to low doses of a mutagenic or clastogenic agent, they often become less susceptible to a higher dose administered subsequently[1] discovered that human lymphocytes treated with 3H-TdR showed an adaptive response to radiation can be observed in other mammalian cells including murine germ cells,[2] mouse bone marrow cells,[3] cultured mouse SR-1 cells,[4] and C3H 10T1/2 cells.[5] Even though their mechanisms have not been clarified, one possible reason is that radiation causes heterogeneous DNA damage including DNA strand breaks, base damage and cross-linking, and low dose pretreatment reduces these damages. Such adaptive responses are also generated in response to a wide range of stresses including alkylating agents,[6] bleomycin,[7] and H_2O_2.[8] One report suggested that an adaptive response, as assessed by cell viabilit, occurred in normal cells but not in cancer cells.[9]

Apoptotic cell death in its earlier stages is characterized by cellular shrinkage, marked condensation, and endonuclease cleavage of DNA, and in its end stages, by nuclear and cellular fragmentation.[10,11] In this study, we examined the induction of adaptive response by pretreatment with low-dose (0.01 Gy) radiation in normal and neopalstic cells, and the reduction of apoptosis as one mechanism of adaptive response.

MATERIALS AND METHODS

1) Cells and Culture

Using the Ficoll-paque gradient method, normal mouse lymphocytes (NL) were prepared as described previously from 6-8-week-old C57BL/6 mouse spleen.[12] Briefly, mouse spleen was cut into several pieces and minced by tapping with a syringe plunger in Hanks balanced salt solution (HBSS). Mononuclear cells were prepared by centrifugation through Ficoll-hypaque solution. Cells were rinsed twice with HBSS and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids, 5 mM β-mercaptoethanol, and antibiotics. L929 cells from murine connective tissue were grown in Dulbeccos minimum essential medium (MEM) containing with 10% FBS and antibiotics.[12] Lymphoma cells (EL-4, L5178Y-R, and L5178Y-S) were obtained from ATCC (Rockville, MD, USA) and cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics. L5178Y- R (Y-R) is resistant to X-rays and sensitive to ultraviolet, heat and certain potential anticancer drugs, and L5178Y-S (Y-S) is an X-ray-sensitive strain.[13,14] Primary mouse keratinocytes (PK) were isolated from Balb/c mouse and were grown in Eagles MEM with 8% Chelex- treated FBS and 0.2% penicillin/ streptomycin solution (GIBCO, BRL, Gaithersburg, MD, USA). Media at a concentration of 0.05 mM Ca^{2+} maintained a basal cell-like population of undifferentiated cells.[15] For the inroduction of a *va-ras*^{Ha} gene into primary keratinocytes (ras-PK), cultured mouse keratinocytes were washed with PBS and incubated with virus at a multiplicity of infection of 1 : 1, in a total volume of 0.5 ml/60 mm dish in EMEM in the presence of 4 μg/ml polybrene (Sigma, St., Louis, MO). After 1 ~ 1.5 hr, 2.5 ml of EMEM was added to replace with fresh virus-free EMEM for 48 ~ 72 hr and cultured 4 ~ 6 days before use. Line 308 papilloma cells were derived from 7,12-dimethyl- benz(a) anthracene-initiated Balb/c mouse epidermis and selected for resistance to Ca^{2+}-induced differentiation. This cell line contains an activating mutation in the c-Ha-*ras* gene and produces benign tumors when grafted into nude mouse.[16]

2) Irradiation

In monolayer cell culture, cells were plated in sterile 10 cm dishes and incubated at 37°C under

humidified, 5% CO_2–95% air in culture medium until 70%~80% confluent; 5~10×10^4 cells per ml media were plated in suspension cells before irradiation. High dose irradiation was by gamma rays from ^{60}Co theratron-780 (Atomic Energy of Canada, Ltd., Canada) at a dose rate of 1.394 Gy/min, while 137 Cs irradiator at a dose rate of 0.143 cGy/min was used for low dose irradiation.

3) Survival and clonogenicity assays

Cells were maintained in a state of logarithmic-phase growth. The viability of NL, Y-S, Y-R, PK, ras-PK, and EL-4 after irradiation was determined by the trypan blue dye exclusion method; 308 and L929 cells were resuspended in media after irradiation, and seeded in 10 cm culture plates at a fixed density of 1,000 cells/plate. Cultures were maintained for 15~17 days, and the formation of colonies was scored in triplicate using a colony counter (Imaging Product International Inc., USA).

4) Sandwich enzyme immunoassay

After irradiation, cells were incubated for the times indicated and then harvested by centrifugation at 200 g for 5 min. The pellet was rinsed with 1 ml of culture medium and lysed with 1 ml of incubation buffer (Böeringer Mannheim, GmbH, Germany) for 30 min at 4°C. Cell lysates were centrifuged at 10,000 g for 10 min and the supernatant was saved carefully for sandwich enzyme immunoassay; this was performed using a Cell Death Detection Kit (Böeringer Mannheim, GmbH, Germany).

5) Detection of DNA ladder

Cells (1~1.5×10^6) including floating cells in the medium were harvested and washed with PBS after irradiation. They were then lysed in lysis buffer [25 mM Tris (pH 7.5), 0.5% SDS, 5 mM EDTA, and 1 mg/ml of proteinase K] at 50°C for 4 hr. DNA was extracted with an equal volume of phenol/chloroform and was then precipitated overnight at -20°C with sodium acetate at a final concentration of 0.3M and 2 volumes of ethanol. The pellet was washed with 70% ethanol and dried at room temperature; the precipitated DNA resuspended in TE [10 mM Tris, 1 mM EDTA (pH 8.0)] was incubated with 200 mg/ml of RNase for 30 min at 37°C, and separated by electrophoresis through 1.5% agarose gel.

6) TUNEL assay

After irradiation, cells were fixed in 95% ethanol and apoptosis was morphologically analysed using an Apoptag assay kit (Oncor Inc., Gaithersburg, MD, USA). TUNEL positive cells were visualized by the anti-horseradish peroxidase-based detection method, which produces a dark brown color; cells were counter-stained with 0.5% (w/v) methyl green in 0.1M sodium acetate (pH 4.0). At least 100 cells were scored for each determination.

7) Statistics

The points shown in figures are the means of three independent determinations. Students t-test was used for statistical analysis.

RESULTS

1) Survival curves

The survival fractions of normal or neopalstic cells after irradiation by various doses of γ-rays are shown in Fig. 1. Living cells of NL, PK, Y-S, ras-PK, Y-R, and EL-4 were counted by the trypan blue dye exclusion method and those of L929 and 308 by colony forming assay. Y-R cells, resistant to X-rays, were significantly resistant to γ-rays, Y-S cells, however, were sensitive to γ-rays (Fig. 1D). Sixteen and 48 hr after irradiation, ras-PK, *v-ras*^{Ha}- transfected primary keratinocyte, was also more resistant to γ-rays than PK (Fig. 1B). High-challenging doses were selected from the survival curves as doses that induced almost 50% cell death in each cell type. However, for high-challenging doses of Y-R or ras-PK, the doses chosen were the same as doses of Y-S or

PK, even though these doses induced less than 50% cell death: 19.4% in Y-R and 30.4% in ras-PK, respectively.

2) Determination of adaptive response

Adaptive response was assessed by cell viability. Because previous reports have stated that the optimal interval for induction of adaptive response in NL was 4 hr,[12] the response was examined with both 4 hr and 7 hr interval between the priming dose of 0.01 Gy and the high-challenging dose, which was as follows: 2 Gy in NL; 8 Gy in PK and ras-PK; 3 Gy in Y-R, Y-S, EL-4, and 308; and 4 Gy in L929. Relative viability was examined 24 hr after high challenging dose irradiation; when pretreated with low dose radiation, that of adapted NL, PK, Y-R, and L929 cells was significantly higher than that of cells irradiated only with a high challenging dose. In NL, PK, and Y-R, the survival fraction of adapted cells was greater with 4 hr interval than with 7 hr interval. In the case of L929 cells, the converse was true (Fig. 2a). This result indicates that the optimal time interval for the induction of adaptive response varied depending on cell type (Fig. 2b).

3) Quantitation of apoptosis

To determine the mode of cell death resulting from radiation, apoptosis was investigated using sandwich enzyme immunoassay in these cells and an alternative method of apoptosis detection involved the use of DNA ladder formation in NL, Y-R, and EL-4, or TUNEL assay in PK, ras-PK, L929, and line 308. In control cells, which were not exposed to γ-rays, apoptosis was induced only in NL, in which a slight degree of spontaneous cell death was induced without radiation (data not shown). Compared with each unirradiated control cell, the extent of apoptotic cell death induced by a high- challenging dose was 23.9% in NL, 136.1% in PK, 41.4% in Y-R, 18% in L929, 31.4% in ras-PK, 240.4% in Y-S, and 60.1% in EL-4. Pre-exposure to 0.01 Gy of radiation significantly reduced cell death in NL, PK, L929, and Y-R (Fig. 3a). In contrast with these results, the reduced cell death in ras-PK, Y-S, EL-4, and 308 could not be observed (Fig. 3b). In the case of ras-PK, a slight reduction of apoptosis was observed with 4 hr interval, but it was not significant (Figs. 3b and 4b). The result of DNA ladder formation or TUNEL assay was similar to that shown by sandwich enzyme immunoassay; in NL, PK, Y-R, and L929, apoptosis was reduced by induction of adaptive response (Fig. 4a), but in ras-PK, EL-4, and 308 cells, this was not so (Fig. 4b). When TUNEL assay of PK was performed with 4 hr or 24 hr interval instead of 7 hr interval, apoptosis was reduced by induction of adaptive response significantly in cells with 4 hr interval, but not with 24 hr interval (Fig. 4aA).

DISCUSSION

This study provides evidence that an adaptive response to ionizing radiation is induced by a dose of 0.01 Gy in normal cell types such as NL, PK, and L929, but not in neoplastic cells such as L5178Y, EL-4, and 308. An adaptive response was induced in lymphoma cells, L5178Y-R because these cells had already adapted to X-rays and their signal transduction pathways and regulating genes might already have been changed. Similarly, RIF (radiation induced mouse fibrosacoma) cells showed no adaptive response, though this response was shown by TR, their corresponding thermoresistant line (data not shown). In addition, the result that an adaptive response in keratinocytes was diminished in tendency according to the carcinogenesis stage (PK, ras-PK, and 308) was also supported this. In normal (NL, PK, and L929) or pre-adapted cells (Y-R), pretreatment with low-dose radiation increased cell survival when subsequent high-challenging dose radiation was administered 4 hr or 7 hr later. These cells usually showed maximum resistance to ionizing radiation when the priming

dose was given 4 hr prior to the high- challenging dose, but in L929 cells, 7 hr interval provided maximum resistance (Fig. 2). These results suggest that the time interval required for the induction of adaptive response differs according to cell type. It has been reported that an adaptive response was usually induced in normal cells such as murine germ cells[2], mouse bone marrow cells,[3] C3H 10T1/2 cells,[5] mouse lymphocyts,[12] human lymphocytes,[17] and human normal fibroblast.[9] Our results also suggested that pretreatment with low-dose radiation induced an adaptive response only in normal cells. Ishii *et al.*[9] suggested that this response was induced by gap junctional intercellular communication. Since this is deficient in malignant cells, an adaptive response was not induced.[18,19] In contrast, an adaptive response in neoplastic cells has been reported[20]; human malignant cells were used and pretreatment involved a dose of 0.05 Gy/day over a 4-day period. It is not at present understood why an adaptive response was not induced in neoplastic cells; one possible explanation is that since these cells show changes in gene/protein expression, a low-dose of 0.01 Gy may not be sufficient to induce an adaptive response. While the priming dose of 0.01 Gy was the lowest dose of radiation in this system. Alternatively, this response may be influenced by the dose rate.[21] Finally, intervals of 4 and 7 hr between the priming and high-challenging dose may not be appropriate for the induction of an adaptive response in neoplastic cells. The mechanisms of, and conditions for, adaptive response to radiation have not been clarified, though one possible explanation relates to the induction of DNA repair processes in response to low-doses of around 0.01 Gy. The induction of new proteins in response to low-doses is regarded as experimental support for this explanation[8] and it could be caused by the effect of low-doses on chromatin conformation near genes coding for DNA repair proteins. As a result of changes in its conformation, chromatin may be less sensitive to damage by the indirect effect of a challenge dose,[22] or this reduced sensitivity may be due to increased accessibility of damaged sites to repair enzymes.[23] We have previously suggested that in mouse lymphocytes, reduced apoptosis is another mechanism of adaptive response.[12] When we confirmed that using several apoptosis detection methods in normal and neoplastic cells, apoptosis was inhibited by pretreatment with low-dose radiation in normal and pre-adapted cells, but not in neoplastic cells (Figs. 3 and 4). These results were similar to those concerning cell viability and suggested that in normal and pre-adapted cells, reduced apoptotic cell death might be a mechanism of adaptive response induced by low-dose radiation.

In conclusion, the data presented indicate that pretreatment with low-dose (0.01 Gy) radiation enhances the radio-resistance, as assessed by cell viability, in normal or pre-adapted, but not in neoplastic cells. In addition, it is suggested that an apoptosis pathway is involved in this adaptive response.

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Fig. 1. Cell survival curves: Survival curves were obtained from normal lymphocytes (A, NL), mouse primary keratinocytes (PK) and *v-ras*Ha-transfected keratinocytes (ras-PK) (B), L929 (C), L5178Y-S (Y-S), L5178Y-R (Y-R) (D), EL-4 (E), and line 308 (F, 308) cells after exposed to various doses of radiation. Viable cells of NL, Y-R, Y-S, and EL-4 cells were counted by the trypan blue dye exclusion method at 24 hr after irradiation, while PK and ras-PK were at 16 hr (---) and 48 hr (-). Those of L929 and 308 cells were counted by colony forming assay. At the indicated times, Y-R and ras-PK were significantly resistant to γ-rays compared to Y-S or PK, respectively ($*P < 0.05$).

Fig. 2. Determination of adaptive response: (a) Normal lymphocytes (A, NL), mouse primary keratinocytes (B, PK), L5178Y-R (C), and L929 (D) cells were irradiated to 0.01 Gy. After 4 or 7 hr, high-challenging doses of 2 Gy for NL, 8 Gy for PK, 4 Gy for L929, and 3 Gy for Y-R were administered. Cell viability was tested 24 hr after high-challenging doses, except for NL, which was tested at 6 hr after high-challenging irradiation. All cells were counted by the trypan blue dye exclusion method. (b) *v-ras*^{Ha}-transfected keratinocytes (A, ras-PK), L5178Y-S (B), EL-4 (C), and 308 (D) cells were irradiated to 0.01 Gy. After 4 or 7 hr, high challenging doses of 8 Gy for ras-PK and 3 Gy for Y-S, EL-4, and 308 were administered. Viable ras-PK, Y-S, and EL-4 cells were counted at 24 hr by the trypan blue dye exclusion method, while those of 308 were counted by colony forming assay. Significant difference was found when compared to cells irradiated only with high challenging dose ($*P < 0.05$). 1, Cells irradiated only with high-challenging dose; 2, adapted cells with 4 hr interval between priming and high-challenging dose radiation; 3, adapted cells with 7 hr interval between priming and high-challenging dose radiation.

Fig. 3. Quantitation of apoptosis using sandwich enzyme immunoassay: (a) Normal lymphocytes (A, NL), mouse primary keratinocytes (B, PK), L5178Y-R (C), and L929 (D) cells were irradiated to 0.01 Gy. After 4 or 7 hr, high-challenging doses of 2 Gy for NL, 8 Gy for PK, 4 Gy for L929, and 3 Gy for Y-R were administered. (b) *v-ras*^{Ha}-transfected keratinocytes (A, ras-PK), L5178Y-S (B), EL-4 (C) cells were irradiated to 0.01 Gy. After 4 or 7 hr, high challenging doses of 8 Gy for ras-PK and 3 Gy for Y-S, EL-4 cells were administered. Using sandwich enzyme immunoassay, apoptosis induction was detected at 24 hr after high-challenging dose radiation except for NL, which was tested at 6 hr after this dose. A significant difference was found when compared to the cells irradiated by high-challenging dose only ($*P < 0.05$). 1, Cells irradiated only with high-challenging dose; 2, adapted cells with 4 hr interval between priming and high-challenging dose radiation; 3, adapted cells with 7 hr interval between priming and high-challenging dose radiation.

Fig. 4. Quantitation of apoptosis using DNA ladder or TUNEL assay: (a) Normal lymphocytes (A, NL), mouse primary keratinocytes (B, PK), L5178Y-R (C), and L929 (D) cells were irradiated to 0.01 Gy. After 4 or 7 hr (in the case of PK, 24 hr), high- challenging doses of 2 Gy for NL, 8 Gy for PK, 4 Gy for L929, and 3 Gy for Y-R were administered. Using DNA ladder for NL and Y-R or TUNEL assay for PK and L929, apoptosis induction was detected at 24 hr after high-challenging dose radiation except for NL, which was tested at 6 hr after this dose. (b) *v-ras*^{Ha}-transfected keratinocytes (A, ras-PK), EL-4 (B), and 308 (C) cells were irradiated to 0.01 Gy. After 4 or 7 hr, high-challenging doses of 8 Gy for ras-PK, 3 Gy for EL-4 and 308 were administered. Using DNA ladder for EL-4 or TUNEL assay for ras-PK and 308, apoptosis induction was detected at 24 hr after high-challenging dose radiation. A significant difference was found when compared to the cells irradiated by high-challenging dose only ($*P<0.05$). 1, Unirradiated control cells; 2, cells irradiated only with high-challenging dose; 3, adapted cells with 4 hr interval between priming and high-challenging dose radiation; 4, adapted cells with 7 hr interval between priming and high-challenging dose radiation.