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Pretreatment of Low Dose Radiation Reduces Radiation-Induced Apoptosis in Mouse Lymphoma (EL4) Cells

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Induction of an adaptive response to ionizing radiation in mouse lymphoma (EL4) cells was studied by using cell survival fraction and apoptotic nucleosomal DNA fragmentation as biological end points. Cells in early log phase were pre-exposed to low dose of γ-rays (0.01 Gy) 4 or 20 hrs prior to high dose γ-ray (4, 8 and 12 Gy for cell survival fraction analysis; 8 Gy for DNA fragmentation analysis) irradiation. Then cell survival fractions and the extent of DNA fragmentation were measured. Significant adaptive response, increase in cell survival fraction and decrease in the extent of DNA fragmentation were induced when low and high dose γ-ray irradiation time interval was 4 hr. Addition of protein or RNA synthesis inhibitor, cycloheximide or 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (DRFB), respectively during adaptation period, the period from low dose γ-ray irradiation to high dose γ-ray irradiation, was able to inhibit the induction of adaptive response, which is the reduction of the extent DNA fragmentation in irradiated EL4 cells. These data suggest that the induction of adaptive response to ionizing radiation in EL4 cells required both protein and RNA synthesis.

Key words: Low dose, Adaptive response, DNA fragmentation, Mouse lymphoma cells

INTRODUCTION

Pretreatment of low dose radiation such as tritiated thymidine (Olivieri *et al.*, 1984; Wienke *et al.*, 1986) or low doses of X-irradiation (Shadley and Wolff, 1987; Shadley *et al.*, 1987) induced adaptive response, thus result in a reduction in the yield of chromosomal aberrations by subsequent high doses of X-rays in human lymphocytes. This phenomenon is similar to the induction of a new pathway for DNA repair in *E. coli* (Samson and Cairns,1977), which has been called an adaptive response. It has been reported that adaptive response is inhibited by the presence of poly (ADP-ribose) polymerase inhibitor, 3-aminobenzimide (Wienke *et al.*, 1986; Shadley and Wolff, 1987). However, the mechanism of the induction of adaptive response to ionizing radiation has not been elucidated yet.

It is widely known that ionizing radiation directly or indirectly induces damages in biologically important macromolecules such as DNA in cells. Therefore, the extent of chromosomal aberrations was used as a biological endpoint of radiation damages. Among several analytical methods of chromosomal aberrations, chromatid and isochromatid breaks (Shadley and Wolff, 1987; Shadley *et al.*, 1987; Wolff *et al.*, 1988; Bosi and Olivieri, 1989; Shadley and Wiencke *et al.*, 1989; Cai and Liu, 1990; Cortes *et al.*, 1990) and micronucleus formation (Fenech and Morley, 1985; Ikushima, 1987; Azzam *et al.*, 1994; Ono *et al.*, 1994) were used as biological end points in many cases. These double-strand DNA lesions produced by ionizing radiation, leading to chromosome aberrations result in cell death. Recently, it was reported that some cells undergo apoptosis upon exposure to ionizing radiation (Radford *et al.*, 1994; Bump *et al.*, 1994). Apoptosis is morphologically characterized by cellular shringkage, chromatin condensation, and nuclear fragmentation. During apoptosis, double strand cleavage occurs at the linker regions between nucleosomes to produce DNA fragments that are multiples of approximately 185 bp (Willie, 1980; Umansky, 1982; Arends *et al.*, 1990) which develop characteristic DNA ladder pattern on agarose gels.

In this study, we examined the induction of adaptive response to ionizing radiation in mouse lymphoma (EL4) cells by pretreatment of low dose γ-ray. We used cell survival fraction and the extent of DNA fragmentation as biological end points of radiation

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damages. In addition, to investigate the possible mechanism of the induction of adaptive response, we inhibited protein or RNA biosynthesis and observed the induction of adaptive response to ionizing radiation in EL4 cells.

MATERIALS AND METHODS

Cell culture and γ-ray irradiation

Mouse lymphoma (EL4, ATCC TIB39) cells were grown in a humidified 5% CO_2/95% air in an incubator at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sod. pyruvate, nonessential amino acids and antibiotics. EL4 cells were grown to reach early log phase (5×10^5 cells/ml) and irradiated with γ-rays from ^{137}Cs irradiator with dose rate of 0.143 cGy/min (low dose) and/or from a ^{60}Co source (Theratron-780 teletherapy unit) yielding a dose rate of 1.394 Gy/min (high dose). Number of viable and dead cells were enumerated by tryphan blue exclusion test.

Purification of DNA

Cells were harvested 24 hrs after irradiation by centrifugation at 800×g for 5 min. Pellets were rinsed with ice-cold phosphate buffered saline (PBS) and resuspended in 0.01 vol. of TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). Cells were lysed with 10 vol. of lysis buffer (0.5% SDS, 25 mM Tris-Cl, pH 7.5, 5 mM EDTA). Proteinase K was added to the sample to a final concentration of 1 mg/ml and incubated at 50°C at least for 3 hrs. Samples were extracted with phenol:chloroform (1:1) 2-3 times and once with chloroform. DNA's were precipitated with 2.5 vol. of ethanol, washed and dried with common methods (Sambrook *et al.*, 1989), and then redissolved in 100 μl of TE buffer. RNase A was added to the sample to a final concentration of 200 μg/ml and samples were incubated for 30 min at 37°C and 5 min at 65°C. DNA was subjected to electrophoresis in 1.5% agarose gel and visualized with ethidium bromide. Results shown are an example of 3 different experiments.

Sandwich enzyme immunoassay

Cells were grown and irradiated as above except that cells were labeled with 5-bromo-2'-deoxy-uridine (BrdU, final conc. 10 μM) for about 18 hrs. After irradiation, cells were lysed by adding lysis buffer. Cell lysate was centrifuged for 10 min at 250×g and supernatant was removed for sandwich enzyme immunoassay. Immunoassay was performed with Cellular DNA fragmentation ELISA kit (Boeringer Mannheim) as recommended by the supplier.

Drug treatment

Cycloheximide was made in 50% ethanol and 50% phosphate buffered saline (PBS) solution. 5,6-Dichloro-1-β-d-ribofuranosylbenzimidazole (DRFB, Fluka) was dissolved in absolute ethanol at a concentration of 0.1 M and was sonicated before use. Final concentrations of ethanol in cell culture media was lower than 0.5%. Equivalent quantities of ethanol was added to controls. Drugs were added to cell culture media immediately after low dose γ-ray irradiations at concentrations indicated in result section and incubation was continued as described above. Prior to high dose γ-ray irradiations, cells were pelleted by centrifugation and supernatant was removed. Cell pellets were rinsed with PBS and cells were resuspended in fresh media.

RESULTS

Induction of adaptive response in EL4 cells

In order to determine the optimal period of induction of adaptive response to ionizing radiation in mouse lymphoma cells, cells were irradiated with low dose γ-ray (0.01 Gy), then irradiated with 0, 4, 8 and 12 Gy of high dose γ-rays with 4 and 20 hr time intervals. Twenty four hours after irradiation of cells with high dose γ-rays, cell survival fractions were calculated from the numbers of viable and dead cells (Fig. 1). Adaptive response, increase in cell survival fraction, was observed in both low dose-γ-ray-pretreated groups with 4 or 20 hr prior to high dose γ-ray irradiation at all high dose ranges (4-12 Gy) ex-

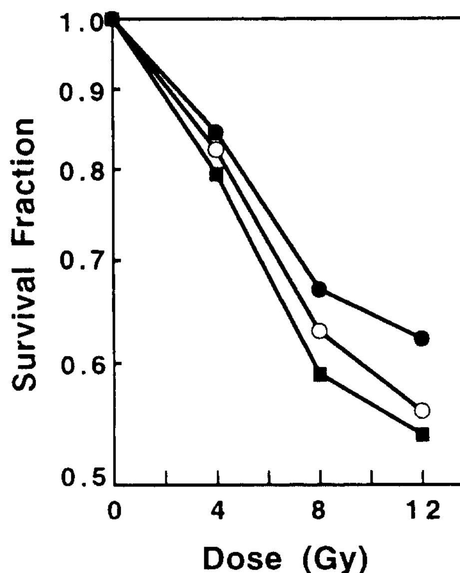

Fig. 1. Survival fractions for EL4 cells after γ-ray irradiations. Cells were irradiated with 0.01 Gy of low dose γ-rays 4 hr (●) or 20 hr (○) prior to high dose γ-ray radiations or with high dose γ-ray only (■). Results shown are the average values from three different experiments.

amined in this study with various extent. Most significant increase in cell survival was observed when cells were irradiated with low dose γ-ray 4 hrs before 8-12 Gy of high dose γ-ray irradiation.

Reduction in radiation-induced apoptosis in low dose radiation-adapted EL4 cells

Ionizing radiation caused internucleosomal DNA fragmentation which is one of the characteristics of apoptosis in EL4 cells (Fig. 2). Low dose γ-ray (0.01 Gy) treatment alone did not induce apoptosis in EL4 cells. Pretreatment of low dose γ-ray 4 hr prior to high dose (8 Gy) irradiation reduced DNA fragmentation remarkably. Pre-exposure to low dose γ-ray 20 hr prior to high dose irradiation reduced DNA fragmentation slightly but it was not significant. It seems that the adaptive response induced by low dose treatment diminished within 20 hr after low dose treatment. The extent of DNA fragmentation was quantitated by sandwich enzyme immunoassay (Fig. 3). High dose γ-ray-irradiated cells showed extensive DNA fragmentation compared to that of unirradiated control cells. When low dose γ-ray was pretreated 4 hrs prior to high dose irradiation, the extent of DNA fragmentation was decreased almost to half of that of high dose γ-ray-irradiated cells.

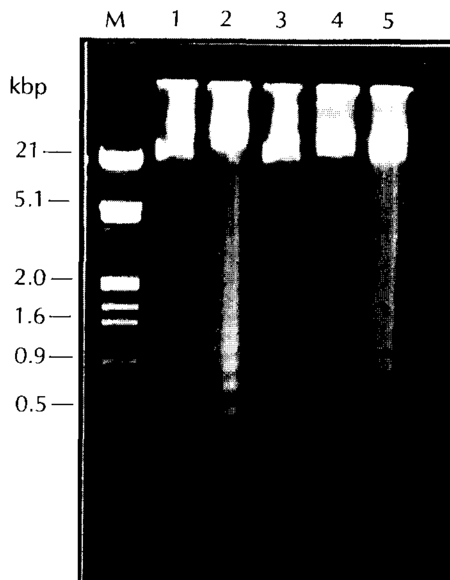

Fig. 2. DNA fragmentation analysis of low dose γ-ray-adapted EL4 cells. Lane 1, unirradiated control; lane 2, irradiated only with 8 Gy of high dose γ-ray; lane 3, irradiated only with 0.01 Gy of low dose γ-ray; lanes 4 and 5, cells were irradiated with 0.01 Gy of low dose γ-rays 4 (lane 4) or 20 hr (lane 5) prior to 8 Gy of high dose γ-ray irradiation. DNA was isolated 24 hr after low dose (lane 3) or high dose (lanes 2, 4 and 5) γ-ray irradiations and analyzed on 1.5% agarose gel.

Biosynthesis of protein and RNA are required to induce adaptive response in EL4 cells

We tested the possible effect of protein or RNA synthesis inhibition by adding cycloheximide or DRFB to cell culture media during adaptation period. Protein synthesis inhibitor, cycloheximide was added to the culture media immediately after low dose γ-ray irradiation and the media was replaced with fresh media, and the extent of DNA fragmentation was observed (Fig. 4A). When no cycloheximide was present in culture media, almost no DNA fragmentation was observed after high dose γ-ray irradiation in low dose radiation-adapted EL4 cells. At low concentrations of cycloheximide (2 μg/ml), DNA fragmentation was slightly detected. At a concentration of 5 μg/ml of cycloheximide, the extent of DNA fragmentation after high dose γ-ray irradiation in low dose radiation-adapted cells was almost same as that of high dose γ-ray only-irradiated cells (compare lane 3 and 7 in Fig. 4A). Therefore, the induction of adaptive response was blocked at 5 μg/ml of cycloheximide. At higher concentrations of cycloheximide (above 10 μg/ml), the extent of DNA fragmentation was even greater than that of high dose γ-ray-irradiated cells. Possibly, most of cells underwent apoptosis after high dose γ-ray irradiation at this condition. In order to study the effect of RNA synthesis inhibition, a reversible RNA synthesis inhibitor, DRFB (Sellins and Cohen, 1987) was added to the cell culture media during adaptation period (Fig. 4B). Almost no DNA fragmentation was detected after high

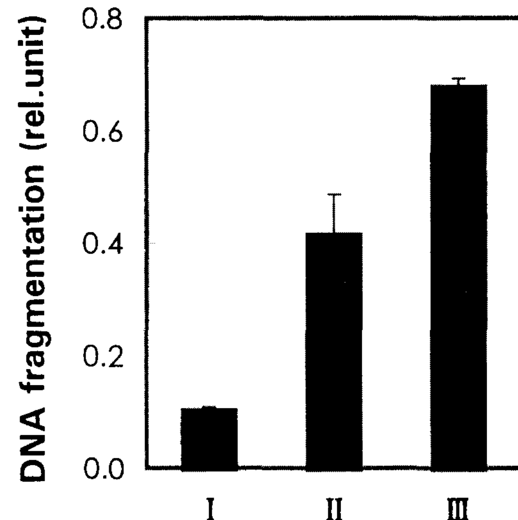

Fig. 3. Quantitation of DNA fragmentation by sandwich enzyme immunoassay. EL4 cells were unirradiated (I) or preexposured to 0.01 Gy of low dose γ-ray 4 hr prior to 8 Gy of high dose γ-ray irradiation (II) or irradiated with 8 Gy of high dose γ-ray (III). Cells were harvested 24 hr after high dose γ-ray irradiation (II and III) and analyzed by ELISA. Values are the means±SD (n=3).

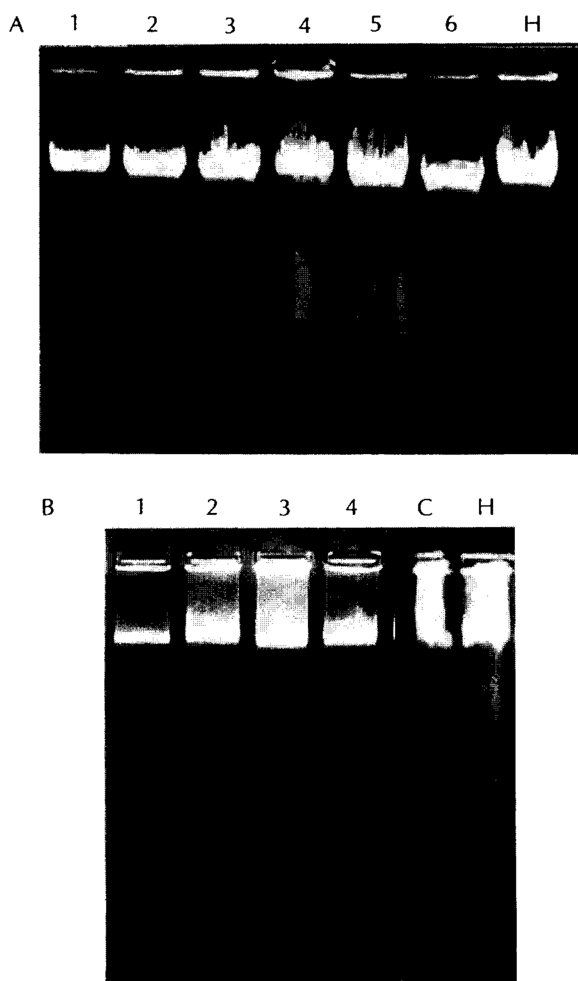

Fig. 4. Effect of protein and RNA synthesis inhibitors on nucleosomal DNA fragmentation in low dose radiation-adapted EL4 cells. EL4 cells were pre-exposured to 0.01 Gy of low dose γ-ray 4 hr prior to 8 Gy of high dose γ-ray irradiation. A. Cells were incubated with 0, (lane 1), 2 (lane 2), 5 (lane 3), 10 (lane 4), 20 (lane 5), or 50 μg/ml (lane 6) of cycloheximide during adaptation period (from immediately after low dose γ-ray irradiation to right before high dose γ-ray irradiation). H, 8 Gy of high dose γ-ray-irradiated cells. B. Cells were incubated with 0 (lane 1), 62.5 (lane 2), or 125 μM (lane 3) of 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (DRFB) during adaptation period. C, unirradiated control; H, 8 Gy of high dose γ-ray-irradiated cells.

dose γ-ray irradiation in low dose radiation-adapted EL 4 cells in the absence of DRFB. In the presence of DRFB at concentrations of 62.5-250 μM during adaptation period, characteristic DNA ladder pattern was observed. The extent of DNA fragmentation were most pronounced at a concentration of 125 μM of DRFB. The induction of adaptive response, which is inhibition of DNA fragmentation was blocked by RNA synthesis inhibitor, DRFB, at concentrations ranged from 62.5 to 250 μM. Our results showed that inhibition of induction of DNA fragmentation in low dose γ-ray pre-exposured-EL4 cells was blocked in the presence of either protein or RNA synthesis inhibitors. These data suggest that both protein and RNA synthesis are required to induce adaptive response to ionizing radiation in EL4 cells.

DISCUSSION

Our data described in this study demonstrate an adaptive response to ionizing radiation is induced in mouse lymphoma (EL4) cells. Pretreatment of low dose γ-ray irradiation increased cell survival fraction caused by subsequent high dose γ-ray irradiation in EL4 cells. Cells showed maximum resistance to ionizing radiation when the low dose radiation was given 4 hrs prior to high dose radiation. Variations exist with respect to the time intervals needed to express the effect of low dose radiation. However, a 4 hr time interval was sufficient to induce adaptive response (Ikushima, 1987; Shadley *et al.*, 1987; Kim *et al.*, 1995a; Kim *et al.*, 1995b; Kim *et al.*, 1996). It seems that low dose radiation-induced adaptive response persist about 20 hrs in EL4 cells. These findings are in consistence with our previous reports (Kim *et al.*, 1995a; Kim *et al.*, 1995b; Kim *et al.*, 1996).

Most important subcellular target of ionizing radiation at biologically relevant dose is the genetic material, DNA. DNA double strand breaks are the critical event in radiation-induced cell killing. Low dose radiation-adapted cells showed decrease in micronucleus formation (Azzam *et al.*, 1994; Kim *et al.*, 1995a; Kim *et al.*, 1995b), chromatid breaks (Bosi and Olivieri, 1989; Shadley *et al.*, 1987; Shadley and Wolf, 1987), and decrease in fraction of apoptotic cells. Several studies suggested the importance of poly (ADP-ribose) polymerase (Shadley and Wolff, 1987; Nelipovich *et al.*, 1988; Soldtenkov *et al.*, 1995) and DNA binding protein (Sanderson and Morley, 1986) in DNA repair. Radiation-induced apoptosis is reported to be accomplished in conjunction with proteolytic cleavage of poly (ADP-ribose) polymerase which catalyzes the transfer of ADP-ribose moiety from NAD to nuclear proteins (Nelipovich *et al.*, 1988; Soldtenkov *et al.*, 1995). In addition, correlation of micronucleus formation and radiation-induced apoptosis was reported (Abend *et al.*, 1995). Therefore, one of the possible mechanisms to induce adaptive response by pretreatment of low dose radiation is the increase in repair of the DNA double strand breaks in these cells, thus result in reduction of apoptotic cells.

Ionizing radiation induced protein biosynthesis in human melanoma (U1-Mel) cells (Boothman *et al.*, 1989). Protein and mRNA synthesis also are induced by UV irradiation of human epidermal keratinocytes (Katasoba, 1988). We previously reported that pretreatment of low dose γ-ray altered protein biosyn-

thesis of high dose γ-ray-irradiated human melanoma cells (Kim *et al.*, 1996). Transcripts of nuclear factor κB and several immediate early genes, *c-fos, c-jun, c-myc* and c-Ha-*ras*, were induced by low dose ionizing radiation (Prasad *et al.*, 1994; Prasad *et al.*, 1995). However, there is no evidence of correlation between these low dose radiation-inducible genes and the induction of adaptive response at present.

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