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Reduction of Radiation-induced Chromosome Aberration and Apoptosis by Dithiothreitol

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We have examined *in vitro* and *in vivo* radioprotective effects of a well-known thiol-containing compound, dithiothreitol (DTT). The treatment of both 0.5 and 1 mM of DTT significantly increased clonogenic survival of γ-ray irradiated Chinese hamster (V79-4) cells. In order to investigate the possible radioprotective mechanism of DTT, we measured γ-ray induced chromosome aberration by micronucleus assay. In the presence of 0.5 mM or 1 mM DTT, the frequencies of micronuclei were greatly reduced in all dose range examined (1.5-8 Gy). Slightly higher reduction in micronucleus formation was observed in 1 mM DTT-treated cells than in 0.5 mM DTT-treated cells. In addition, incubation with both 0.5 and 1 mM of DTT prior to γ-ray irradiation reduced nucleosomal DNA fragmentation at about same extent, this result suggests that treatment of DTT at concentrations of 0.5 and 1 mM reduced radiation-induced apoptosis. *In vivo* experiments, we also observed that DTT treatment reduced the incidence of apoptotic cells in mouse small intestine crypts. In irradiated control group 4.4±0.5 apoptotic cells per crypt were observed. In DTT-administered and irradiated mice, only 2.1±0.4 apoptotic cells per crypt was observed. *In vitro* and *in vivo* data obtained in this study showed that DTT reduced radiation-induced damages and it seems that the possible radioprotective mechanisms of action of DTT are prevention of chromosome aberration.

Key words : Dithiothreitol, Radioprotection, Chromosome aberration, Apoptosis

INTRODUCTION

Because of the increasing use of atomic energy in industries, in hospitals and in research laboratories, the possibilities of human beings being exposured to various radiations become increased. In addition, the radiation doses used in clinics for treatment of disease lesions such as cancer are limited by the tolerance of the normal tissues. Therefore, studies on the effect of radiations on the living organisms, and research and development of radioprotectors are getting more attention.

Since the radioprotective effects of thiols were demonstrated by Patt *et al.* (1949), many thiol-containing compounds were tested for possible radioprotectors (Rèvèsz, 1985). Among them a synthetic aminothiol, WR-2721 [S-2-(3-aminopropylamino) ethylphosphorothioic acid], and its derivative, WR-1065 (the dephosphorylated form of WR-2721), have been shown to decrease radiation-induced damages most effectively (Grdina *et al.*, 1985; Grdina and Nagy, 1986; Smoluk *et al.*, 1988; van der Vijgh and Peters, 1994). The possible radioprotective mechanisms of thiol-containing compounds are presumed to be scavenging of various radicals, depletion of oxygens, reduction of harmful oxidation products, and maintaining the protein sulfahydryls in the proper redox state for enzymatic or chemical repair of DNA damage (Bump *et al.*, 1992; Bump and Brown, 1990; Livesey and Reed, 1987).

Some of non-thiol containing compounds, such as 16,16-dimethyl prostaglandin E_2 ($dmPGE_2$) were reported to have radioprotective ability (Berk *et al.*, 1990). Combined use of radioprotective agents showed synergistic effect. When WR-2721 and $dmPGE_2$ were used together, increased cell survival was observed compared to the effect on cell survival of each agent alone (Hanson, 1987). These results imply that either the radioprotection mechanisms of these two agents are different or they act on different subcellular targets. However, most of radioprotective compounds developed so far are restricted in their use because of their toxic side effects.

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We have studied the mechanism of radiation-induced damages and found the effective radioprotective agents and the mechanisms of action of the possible radioprotective agents (Kim *et al.*, 1995a; Kim *et al.*, 1995b; Kim *et al.*, 1996; Hyun *et al.*, 1997; Kim *et al.*, 1997; Kim, S. G. *et al.*, 1997; Nam *et al.*, 1997; Kim *et al.*, 1998). In this study, we report *in vitro* and *in vivo* radioprotective effect of one of the most well-known thiol-containing compound, DTT and suggest the possible radioprotective mechanism of DTT.

MATERIALS AND METHODS

Cell culture and irradiation

Chinese hamster lung (V79-4, ATCC CCL-93) cells were cultured in DMEM containing 5% fetal bovine serum, 2 mM L-glutamine, and antibiotics in a humidified 5% CO_2/95% air incubator at 37°C. Log phase cells were used for micronucleus assay. Cells were irradiated with indicated doses of ^{60}Co γ-rays (Theratron-780 teletherapy unit) at a dose rate of 1.395 Gy/min at room temperature.

Animals and irradiation

N:GP(s) mice of either sex were caged and allowed free access of pellets of standard food and water. All experiments were performed with 7~8 weeks old mice. Animals were irradiated with 2 Gy of ^{60}Co γ-rays (Gammacell 3000 Elan, Nordion international Co., Canada) at a dose rate of 1.09 Gy/ min.

Clonogenic assay

Cells were irradiated as above with 0, 1, 2, 3, 5, 7, or 9 Gy of ^{60}Co γ-rays. After 7 days of incubation, the cells were fixed in methanol:acetic acid (3:1) and stained with trypan blue. Colonies of more than 50 cells were scored. Plating efficiencies were about 50% for the cell line used.

Micronucleus assay

Cytochalasin-B (Aldrich Chemical Co.) was added to media at a final concentration of 3 μg/ml immediately after irradiation. After 20 hr incubation in the presence of cytochalasin-B, cells were trypsinized and fixed in methanol:acetic acid (3:1) solution. Fixed cells were spread onto slide glasses, air-dried and stained with Giemsa. We used Almássy's method (Almássy *et al.*, 1987) to identify the micronucleus in cytokinesis-blocked binucleated cells and the frequencies of micronuclei were scored in 500 binucleated cells at 400X magnification.

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 irradiations, cells were harvested and 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.

Radiation-induced apoptosis in small intestine

DTT was dissolved in PBS and 1.54 mg of DTT was given to each mouse intraperitoneally at least 30 min before irradiation. Irradiation was performed as described above. Six hours after irradiation, small intestines were removed and fixed in Carnoy's solution for at least 30 min. Histological transverse sections (4 μm thick) were prepared and stained with hematoxylin and eosin. Good longitudinal sections of crypts were selected. With these crypt sections, apoptotic cells were counted as follows. When several apoptotic fragments were, from their size and clustering, thought to represent the remains of a single cell, they were recorded as a single event. Fifty crypt sections were scored for each mouse.

RESULTS AND DISCUSSION

When ionizing radiation is absorbed in biological materials, the radiation may interact with water molecules composing 80% of a cell. As a result of this interaction, the highly reactive hydroxy radicals are formed. It is estimated that about two third of γ-ray damage to DNA in mammalian cells is due to hydroxy radicals. Thus, we used dithiothreitol, which is a well-known thiol-containing antioxidant to evaluate for its radioprotective effect. There are several reports of biphasic cytotoxicity pattern for WR-2721, cysteamine and related thiol-containing compounds: they are more toxic at low concentrations (< 1 mM) than at higher concentrations (> 10 mM) (Mori *et al.*, 1983; Issels *et al.*, 1984; Held and Melder, 1987). Some other reports showed that DTT has radioprotective effect in 1~50 mM concentration ranges (Sölen *et al.*, 1990; Sölen *et al.*, 1991). Thus, we choose concentrations of 0.5 mM and 1 mM of DTT to study *in vitro* radioprotective effect of DTT. First of all, we analyzed cell survival after irradiations in the absence or in the presence of 0.5 mM or 1 mM of DTT (Fig. 1). The number of survival fractions was calculated and the survival curve obtained by fitting the fractions to the linear quadratic model. As shown in Fig. 1, in the presence of 0.5 mM or 1 mM DTT, cell survival curves were obviously shifted to the right which means that cell survivals in these conditions are significantly

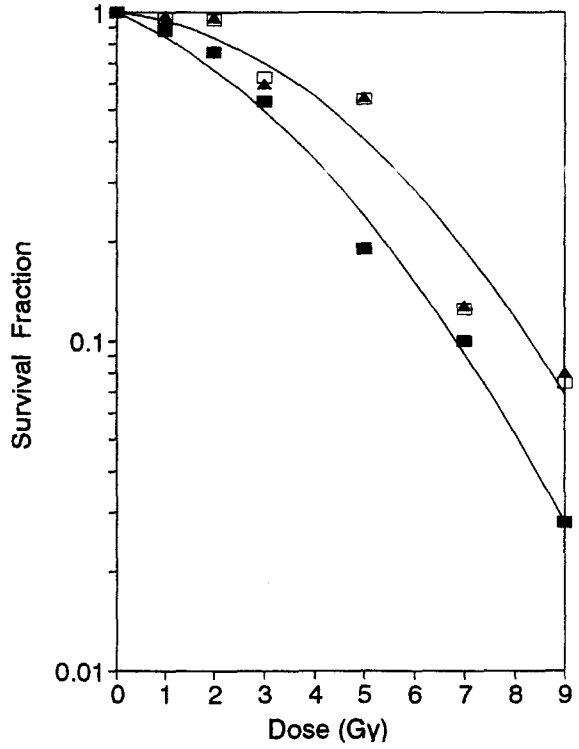

Fig. 1. Survival curves of Chinese hamster lung (V79-4) cells after γ-ray irradiation. Cells were irradiated with γ-rays in the presence of 0 mM (■), 0.5 mM (□) or 1 mM (▲) of DTT.

increased. The expression for the cell survival curve is $SF=Exp-(\alpha D+\beta D^2)$, where SF is the survival fraction of cells at dose D, and α and β are constants. In the absence of DTT, the following equation was obtained; $SF=Exp-(0.15D+0.023D^2)$. In the presence of 0.5 mM or 1 mM DTT, equations $SF=Exp-(0.023D+0.032D^2)$ or $SF=Exp-(0.031D+0.030D^2)$ are obtained respectively. When cells were treated with DTT prior to irradiation, the value of constant α in cell survival curve was significantly increased. It means that DTT treatment decreased cell death caused by one-photon process of radiation. However, there was less significant changes in the value of constant β, this means that there was no such meaningful change in cell death caused by two-photon process of radiation by treatment of DTT.

In order to elucidate the possible mechanisms involved in radioprotective effect of DTT, we have investigated the effect of DTT on the induction of chromosome aberration. Cells were irradiated with γ-ray and cultured for 20 h in the presence of cytokinesis-blocking agent, cytochalasin-B. Then cells were harvested, fixed and frequencies of micronuclei were counted in 500 binucleated cells. Frequencies of micronuclei in the absence or presence of DTT are shown in Table I. In control group, the frequencies of micronuclei were increased along with increase in irradiation dose; at doses of 1.5 Gy, 3 Gy, 6 Gy, and 8 Gy, 7.2%, 9.6%, 13.8% and 24% of micronuclei,

Table I. Micronucleus frequencies of Chinese hamster (V79-4) cells after γ-ray irradiation in the absence or presence of 0.5 mM or 1 mM of DTT

DTT / Dose (Gy)	0 mM		0.5 mM		1 mM	
	Micronuclei in 500 cells	(%)	Micronuclei in 500 cells	(%)	Micronuclei in 500 cells	(%)
0	0	0	0	0	0	0
1.5	36	7.2	28	5.6	15	3.4
3	48	9.6	23	4.6	21	4.0
6	69	13.8	37	7.4	29	5.8
8	117	24	55	11	55	7.4

respectively (Fig. 2). In the presence of 0.5 mM or 1 mM DTT, the frequencies of micronuclei were greatly reduced; at doses of 1.5 Gy, 3 Gy, 6 Gy, and 8 Gy, 5.6%, 4.6%, 7.4% and 11% of micronuclei (0.5 mM DTT) or 3.4%, 4.0%, 5.8%, and 7.4% (1 mM DTT), respectively (Table I and Fig. 2). These data suggest that DTT has protective effect at both concentrations of 0.5 and 1 mM against radiation-induced damages through preventing the induction of chromosome aberration by γ-ray irradiation.

It has been reported that ionizing radiation induces apoptosis (Radford *et al.*, 1994). Thus, we investigated if the extent of radiation-induced apoptosis are reduced by DTT treatment in irradiated cells. We measured the extent of DNA fragmentation, which is one of the characteristics of apoptotic cells (Wyllie, 1980), by sandwich enzyme immunoassay (Fig. 3). When cells were incubated with DTT prior to irradiation, the extent of nucleosomal DNA fragmentation was reduced to

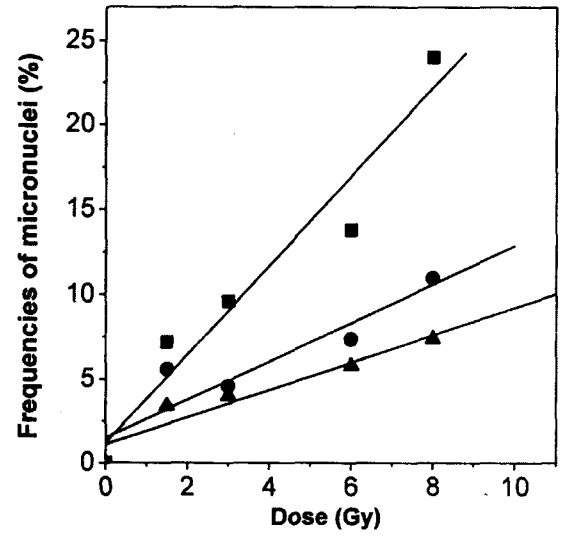

Fig. 2. Dose-response curve for γ-ray induced micronuclei frequencies in Chinese hamster (V79-4) cells. Cells were incubated with 0 mM (■), 0.5 mM (●) or 1 mM (▲) of DTT at least for 30 min, then irradiated with γ-rays as indicated above. Cells were harvested and micronucleus frequencies were analyzed as described in Materials and Methods.

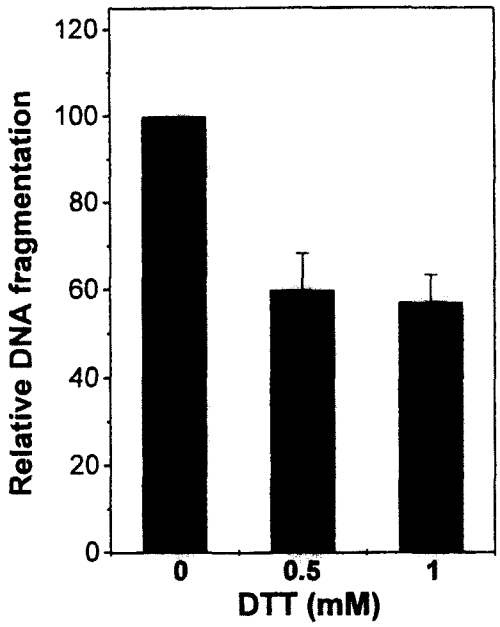

Fig. 3. Quantitation of DNA fragmentation by sandwich enzyme immunoassay. Cells were incubated with 0 mM, 0.5 mM or 1 mM of DTT for at least for 30 min and then irradiated with 10 Gy of γ-ray. Cells were harvested 48 hr after irradiation and analyzed by ELISA. Values are the means±S.D. (n=3).

60.0% (0.5 mM of DTT) or 57.2% (1 mM of DTT), respectively, compared to DTT-untreated and γ-ray irradiated control cells. Thus, it seems that DTT treatment at both concentrations of 0.5 and 1 mM can protect cells from undergoing radiation-induced apoptosis.

Since DTT seemed to protect cells from undergoing apoptosis *in vitro*, we measured the frequencies of apoptotic cells in the small intestinal crypts of 2 Gy of γ-ray irradiated mice and the results are shown in Table II. When there was no treatment 0.09±0.02 apoptotic cells were observed. However, average 4.4 ±0.5 apoptotic cells were observed in each crypt of irradiated mice. When DTT was administered I.P., the average number of apoptotic cells was significantly reduced to 2.1±0.4. These results show that DTT reduced radiation-induced apoptosis *in vivo*.

In this study, we evaluated *in vitro* and *in vivo* radioprotective effect of a well-known thiol-containing antioxidant, DTT. In *in vitro* study, concentrations of DTT used were 0.5 or 1 mM which were reported to be cytotoxic concentration or radioprotective concentration of DTT, respectively. However, our data showed that incubation with DTT at both 0.5 and 1 mM concentrations increased cell survival, decreased micronuclei formation and reduced nucleosomal DNA fragmentation in γ-ray irradiated cells, even though DTT at 1 mM showed slightly higher effects. When DTT was administered to mice, the frequencies of apoptotic cells in small intestinal crypts of γ-ray irradiated mice were significantly reduced. These results suggest that DTT reduces radiation-induced damages *in vivo* and that the possible mechanisms of action of DTT are the prevention of chromosome aberration.

Table II. Effect of DTT on radiation-induced apoptosis in small intestinal crypts of γ-ray irradiated mice

Treatment	Numbers of apoptotic cells
Unirradiated	0.09±0.02
Irradiated	4.4±0.5
DTT, then irradiated	2.1±0.4*
DTT alone	0.04±0.02

DTT was administered I.P. at a dose of 1.54 mg/mouse 30 min prior to 2 Gy of γ-ray irradiations. Two hundred crypts were scored in 4 mice. Mean±S.D., (*, $p < 0.01$).

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