Influence of endogenous glutathione level on X-ray induced cell cycle delay in human lymphocytes

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Abstract. Here, induction of chromosomal aberration after X-irradiation and the pattern of cell cycle kinetics have been investigated in human lymphocytes, after exogenous addition of reduced glutathione or by depleting levels of reduced glutathione endogenously. Involvement of cell cycle regulator proteins such as p53 and p21 has been investigated to elucidate their role in induction of delay in cell cycle progression after irradiation.

INTRODUCTION

With the use of fluorescence plus Giemsa (FPG) staining techniques for the investigation of cell cycle kinetics, it has been established that X-ray-induced cell cycle delay in human lymphocytes, irradiated in the G₀ stage of the cell cycle, is of the order of a few hours after application of 4Gy (Lloyd *et al.* 1977). Cell cycle delay increases with dose (Vulpis *et al.* 1978), showing a linear relationship between the dose delivered and mitotic delay of about 1 h per Gray (Purrott *et al.* 1980). Reduced glutathione (GSH), being an endogenous thiol, has long been thought to affect the sensitivity of cells to radiation (Meister 1983). Earlier studies indicate that certain thiol compounds could remove such radiation-induced delay in cell cycle by restoring the mitotic index in Chinese hamster ovary (CHO) cultures (Yu & Sinclair 1967; Kawasaki 1977). By employing the FPG staining technique, it has been reported that GSH pre-treatment reduced the 2Gy-induced delay in cell cycle, and chromosome aberrations (CAs), significantly but was unable to do so consistently at higher doses of radiation in muntjac lymphocytes (Chatterjee & Jacob-Raman 1986). The primary subcellular target responsible for radiation-induced delay in cell proliferation still remains undetermined. There are reports that damage to DNA (Lucke-Huhle *et al.* 1979), cell division-related proteins (Dewey & Highfield 1976), the cellular and nuclear envelopes (Myers 1970) and cyclic adenosine 3', 5' monophosphate (cAMP) and cyclic guanosine 3', 5' monophosphate (cGMP) metabolism (Charp & Whitson 1978) contribute to the induction of radiation-induced delay. It has been shown earlier that there are equal levels of CA induction after bleomycin (Blem) treatment and after X-irradiation, but cell cycle delay is greater after X-rays than after Blem (Chatterjee & Jacob-Raman 1988). A similar observation was also made by Scott & Zampetti-Bosseler (1985) who measured the G₂ delay induced by Blem and X-rays and

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reported that the induction of this delay is greater after X-rays than after Blem. Such comparative data between X-ray and Blem treatment suggest that the significant cell-cycle delay caused by X-irradiation and its absence after Blem treatment, despite equivalent numbers of chromosomal aberrations, may be caused by factors additional to DNA damage in the case of X-irradiation. It is therefore reasonable to believe that not a single event is responsible for radiation-induced delay in cell cycle kinetics, but it may rather be dependent on multivariant factors.

It has been suggested that human blood cultures have at least two lymphocyte subpopulations with different radiosensitivities (determined by induced CAs) and different rates of progression through the process of DNA synthesis to cell division (Bender & Brewen 1969; Steffen & Michalowski 1973). There are several reports supporting the notion that lymphocytes whose division is delayed are more apt to have aberrant chromosomes than those that enter mitosis early (Boei *et al.* 1996; Hoffmann *et al.* 2002). It has also been demonstrated that buthionine sulfoximine (BSO)-mediated GSH depletion increases radiation induced CAs, with the exception of exchange aberrations (Chattopadhyay *et al.* 1999). However, the influence of such an increased number of CAs on the pattern of cell kinetics after radiation is not known.

Thus, in the present study, the pattern of cell cycle kinetics and the induction of CAs after X-irradiation has been investigated in human lymphocytes after addition of GSH exogenously or depleting the GSH level endogenously. Furthermore, involvement of the cell cycle regulator proteins such as p53 and p21 was investigated to elucidate their role in the induction of delay in cell cycle after irradiation.

MATERIALS AND METHODS

Chemicals

DL-BSO, Hoechst 33258, 5-bromodeoxyuridine (BrdU), Histo-paque, Nonidet P-40, sodium dodecyl sulphate (SDS), aprotinin, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), GSH-reductase and nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma Chemical Company (St. Louis, MO, USA). The culture medium RPMI 1640, foetal calf serum, antibiotics penicillin and streptomycin and mitogen phytohaemagglutinine (PHA) were obtained from Gibco, USA. Giemsa stain was obtained from BDH chemicals Ltd, UK. Other chemicals used in this study were of analytical grade.

Treatment by X-radiation

Peripheral blood was drawn from six healthy male donors and used immediately after venipuncture. All the rules of 'Ethical Guidelines for Biomedical Research on Human Subjects' of the Indian Council of Medical Research, India were followed in all experiments. Lymphocytes were isolated by density gradient centrifugation in Histopaque (Sigma) as described by McFee *et al.* (1997). The monocyte cell layer was washed in phosphate-buffered saline (PBS), and about 1×10^6 viable lymphocytes were added to 1 ml RPMI culture medium in a sterilized small flat bottom 25 ml glass beaker. BSO was dissolved in PBS (pH 7.4) and 5 mM was added to the lymphocytes (in three sets of samples) and kept at 37 °C for 5 h before irradiating them with Faxitron Cabinet X-ray Systems (Model no. 43855D, 110 kVp, 3 mA, Beryllium window thickness 0.76 mm; Faxitron X-Ray Corp, Wheeling, IL, USA) at a dose rate of 1.5 Gy/min. GSH (15 mM) was added in three sets and all six sets of 52 h and 72 h fixed samples respectively, and were kept at 37 °C for 1 h before irradiation (3.5Gy). Lymphocytes from all the six donors were irradiated before and 10 h after PHA-stimulation. Henceforth, they are referred to as G₀- and

G₁-irradiated samples, respectively. For irradiation at the G₁ stage, conditioned medium was removed following centrifugation at 135 g and was kept in a sterilized vial at 37 °C. All the samples were kept at 37 °C for 1 h after irradiation to allow for normal cellular repair before setting up the cultures.

Culture procedure and cell fixation

Cultures were set up in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum. Lymphocytes were stimulated with PHA. BrdU (6 µg/ml) was added to each culture during its initiation. All cultures were incubated at 37 °C and harvested at 52 and 72 h. Colcemid was added at a concentration of 0.01 µg/ml, during the last 3 h, to all cultures. Hypotonic treatment was performed for 18 min, cells were fixed in acetic acid and methanol (1 : 3) and slides were prepared.

Differential staining for sister chromatids

The method of Goto *et al.* (1975) was followed. Slides were incubated for 10 min with Hoechst 33258 dye (50 µg/ml) at room temperature in the dark, were rinsed in distilled water, then mounted in 2xSSC (NaCl-Na-Citrate, pH 6.8) and were kept in sunlight for 30–40 min, depending upon the intensity of sunlight. After rinsing in distilled water, slides were stained in 2% Giemsa for 4 min.

Determination of GSH level in human lymphocytes

The level of total GSH in lymphocytes was estimated by the method of Akerboom and Sies (1981). A total of five blood samples were collected from healthy male donors to act as a control and were also used as a single treatment with BSO. Rules of the 'Ethical Guidelines for Biomedical Research on Human Subjects' of the Indian Council of Medical Research, India were followed in all the experiments. Lymphocytes were separated out from heparinized whole blood on a Ficoll-hypaque density gradient after 5 h of BSO treatment. Freshly collected lymphocytes were washed in ice-cold 0.1 M PBS solution (pH 7.4) and the volume was made up to 1 ml. Cells were counted in a haemocytometer and processed for determination of total GSH level as described earlier (Chattopadhyay *et al.* 1999). Briefly, after deproteinization by 10% ice-cold 5-sulfosalicylic acid, a 50-µl sample suspension was taken and added to 1 ml buffer (0.1 M (ethylenediaminetetraacetic acid) EDTA phosphate buffer, pH 7.0). Then 50 µl NADPH (4 mg/ml), 20 µl DTNB (1.5 mg/ml) and 20 µl GSH reductase (6 U/ml) were added and optical density of the samples was measured at 412 nm using the UV-visible spectrophotometer (Beckman model DU-640). A standard curve was prepared from a stock solution of 1 mM GSH in 5% 5-SSA diluted to 0.1–8 µmol.

Western blot analysis

Expression of p53 and p21 proteins after X-irradiation with or without BSO or GSH treatment was analysed by immunoblotting. Lymphocytes were isolated and washed with PBS as mentioned previously. About 2×10^6 cells were taken in a sterilized, small, flat-bottom 25-ml glass beaker. BSO or GSH treatment was performed, as described previously. Cells were exposed to X-rays (4Gy) at G₀ (before PHA-stimulation) and G₁ (10 h after PHA-stimulation) stages. For irradiation at the G₁ stage of the cell cycle, conditioned medium was removed following centrifugation at 135 g, and was kept in a sterilized vial at 37 °C. All samples were kept at 37 °C for 1 h after irradiation to allow for normal cellular repair before setting up the cultures with PHA. One group of the G₀-irradiated samples was kept without PHA stimulation (G₀-PHA⁻), whereas a further group was treated with PHA (G₀-PHA⁺). Cells were lysed 6 h after irradiation in radioimmunoprecipitation buffer (0.1% SDS, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 50 mM sodium fluoride and 100 U/ml aprotinin). After 30 min of incubation on ice, cell lysates

were centrifuged at 1120 g for 15 min at 4 °C and the amount of protein present was determined using the bicinchonic acid protein assay (Smith *et al.* 1985). An equal amount of protein (80 µg) from each sample was loaded in each well, and equal loading was verified by immunoblotting with actin antibodies (antiactin ACTN05; Neo-Markers, Fremont, USA). Electrophoresis was performed in 12% polyacrylamide separating gel and 5% stacking gel. Proteins were transferred to a 0.45-µm nitrocellulose membrane (Millipore) using Bio-Rad Trans-Blot cell and membranes were probed with 1 : 1000 dilution, mouse monoclonal antibodies against p53 Ab-8 and anti-p21 Ab-6 (Neo-Markers, Fremont, USA), as appropriate. Blots were washed three times for 10 min each in tris-buffered saline Tween 20 (TBST) buffer pH 7.6 (1 M Tris Cl, 5 M NaCl and 0.05% Tween 20) and were incubated with secondary antibody (alkaline-phosphatase conjugated antimouse IgG, 1: 2000; Bangalore Genei, Bangalore, India) for 1 h at room temperature. After extensive washing, the blot was immersed in 4 ml substrate solution of BCIP/NBT (5-bromo-4chloro-3-indolyl phosphate/nitro blue tetrazolium; Bangalore Genei, Bangalore, India). Within 15 min, sufficient staining was obtained. The whole experiment was repeated twice.

Scoring and statistical analysis

Slides were randomly coded. CAs scored in human peripheral blood lymphocytes (PBLs) were from first-cycle metaphases (M1) and were: exchanges including dicentrics and rings (with or without fragments), deletions and chromatid breaks. For scoring cell cycle kinetics, metaphases were categorized as in first, second or subsequent cycles, based on their differential staining patterns. Statistical significance of the difference between control and treated groups for the frequency of M1 cells and aberrant metaphases, was evaluated using 2×2 contingency χ^2 -test, and for different types of aberration simple χ^2 -test was used. The difference of GSH level between BSO treated and untreated groups was evaluated using Student's *t*-test.

RESULTS

Cell cycle progression and CAs were scored simultaneously from all the experiments. However, for simplicity in presentation, cell cycle progression data scored from all the six blood samples in which treatment was given at both G_0 and G_1 cells and fixed at both 52 and 72 h, are presented in Table 1 and CA data, in Table 2.

Cell cycle kinetics

Delay in provision of cell cycle kinetics was measured in terms of increase in frequency of first-cycle metaphases following treatment in comparison to that of untreated controls. Basic cell cycle progression varied among all the six donors and therefore, the percentage of delay induced by X-irradiation in each experiment was measured from the untreated control specimens carried out in parallel. The average delay has been calculated and is presented with the SE. The data presented in Table 1 show that the delay induced in cell cycle progression by 3.5Gy was significant at both G_0 and G_1 stages of the cell cycle, although the extent of delay was more at 72 h (mean delay 25% and 17% at G_0 and G_1 stages, respectively) than in the 52 h (mean delay was 14% and 12% at G_0 and G_1 stages, respectively) samples. Irradiation of BSO-treated G_0 cells showed a tendency to reduction of such delay, whereas such a trend was not distinct when the cells were irradiated while in G_1 . A significant reduction of radiation-induced delay in the cell cycle was observed when the G_0 cells had been pre-treated with GSH. However, GSH pre-treatment to G_1 cells showed poor ability to remove irradiation-induced delay in the cell cycle.

Table 1. Pooled data showing the range of first cycle metaphases (M1) in X-irradiated cultures with or without BSO or GSH and the extent of delay in cell cycle progression

Exptal condtn.	Fixation time (h) (stage)	Total metaphase (no. of expt.)	M1 % $\pm$ SE	M1 range (%)	Mean delay $\pm$ SE
Untreated	52 (G ₀)	700 (4)	77 $\pm$ 4	69–87	–
3.5Gy		700 (4)	91 $\pm$ 2 ^a	87–97	14.3 $\pm$ 3
BSO +3.5Gy		603 (3)	90 $\pm$ 3	85–94	11.0 $\pm$ 6
GSH +3.5Gy		431 (3)	85 $\pm$ 3 ^{a,c}	81–92	5.6 $\pm$ 2
Untreated	52 (G ₁)	479 (4)	80 $\pm$ 4	72–89	–
3.5Gy		392 (4)	92 $\pm$ 1 ^a	89–94	11.5 $\pm$ 3
BSO +3.5Gy		313 (3)	92 $\pm$ 3	89–98	10.0 $\pm$ 2
GSH +3.5Gy		311 (3)	87 $\pm$ 2 ^{a,c}	84–93	4.6 $\pm$ 2
Untreated	72 (G ₀)	731 (6)	25 $\pm$ 3	16–36	–
3.5Gy		900 (6)	50 $\pm$ 5 ^b	36–67	25.2 $\pm$ 3
BSO +3.5Gy		477 (3)	46 $\pm$ 7	31–56	19.3 $\pm$ 5
GSH +3.5Gy		722 (6)	45 $\pm$ 6	29–63	20.0 $\pm$ 6
Untreated	72 (G ₁)	1075 (6)	30 $\pm$ 3	20–40	–
3.5Gy		1065 (6)	51 $\pm$ 5 ^b	38–67	17.3 $\pm$ 3
BSO +3.5Gy		489 (3)	53 $\pm$ 1	51–55	22.0 $\pm$ 2
GSH +3.5Gy		1105 (6)	56 $\pm$ 4	46–71	26.0 $\pm$ 2

^aSignificant at $P < 0.05$ and ^bat $P < 0.01$ 2×2 contingency χ^2 -test compared with respective control; ^cbetween GSH +3.5Gy and 3.5Gy.

Table 2. Pooled data showing the frequency of chromosomal aberrations and aberrant metaphases induced by X-irradiation with or without BSO or GSH

Exptal condtn.	Fixation time (h) (stage)	Total metaphase (no. of expt.)	Percentage of aberrant metaph.	Deletion	Exchanges
Untreated	52 (G ₀)	406 (4)	2 $\pm$ 0.4	0	0
3.5Gy		454 (4)	68 $\pm$ 2	66 $\pm$ 6	46 $\pm$ 1
BSO +3.5Gy		342 (3)	81 $\pm$ 6 ^a	77 $\pm$ 11 ^b	39 $\pm$ 3
GSH +3.5Gy		241 (3)	59 $\pm$ 6 ^a	51 $\pm$ 5 ^b	40 $\pm$ 1
Untreated	52 (G ₁)	432 (4)	3 $\pm$ 1	1 $\pm$ 1	0
3.5Gy		353 (4)	70 $\pm$ 4	63 $\pm$ 5	49 $\pm$ 3
BSO +3.5Gy		256 (3)	80 $\pm$ 5 ^a	76 $\pm$ 12 ^b	39 $\pm$ 4 ^b
GSH +3.5Gy		278 (3)	67 $\pm$ 7	55 $\pm$ 5	47 $\pm$ 4
Untreated	72 (G ₀)	459 (6)	2 $\pm$ 1	0	0
3.5Gy		515 (6)	73 $\pm$ 2	68 $\pm$ 5	48 $\pm$ 2
BSO +3.5Gy		316 (3)	74 $\pm$ 5	75 $\pm$ 4 ^b	37 $\pm$ 3
GSH +3.5Gy		428 (6)	61 $\pm$ 3 ^a	51 $\pm$ 12 ^b	39 $\pm$ 3 ^b
Untreated	72 (G ₁)	440 (6)	2 $\pm$ 1	1 $\pm$ 1	0
3.5Gy		426 (6)	76 $\pm$ 2	71 $\pm$ 4	43 $\pm$ 2
BSO +3.5Gy		337 (3)	80 $\pm$ 4	88 $\pm$ 9 ^b	34 $\pm$ 4 ^b
GSH +3.5Gy		548 (6)	71 $\pm$ 3	53 $\pm$ 6 ^b	50 $\pm$ 2

^aSignificant at $P < 0.05$ 2×2 contingency χ^2 -test compared with respective control; ^b $P < 0.05$ χ^2 -test compared with respective control at d.f. = 2.

Chromosomal aberration

Irradiation (3.5Gy) exposure of these human lymphocytes at both G₀ and G₁ stages induced CAs significantly to a similar extent in the two stages. Frequency of chromatid breaks was very low (data not shown). Frequency of aberrant metaphases was a little more in the 72-h fixed samples than in the 52-h category. Presence of BSO increased radiation-induced deletions and aberrant metaphases but not chromosomal fragment exchanges in both stages of the cell cycle. Pre-treatment with GSH significantly reduced the frequency of radiation-induced aberrant metaphases at G₀ stage, whereas the reduction was poor when cells were irradiated in the G₁ stage. However, the frequency of radiation-induced deletions was reduced significantly and consistently in the presence of GSH in both stages of the cell cycle, whereas for fragment exchange, reduction was observed only in G₀. As a whole, GSH-pre-treatment showed better protection of irradiated cells in G₀ rather than G₁.

Level of reduced GSH

The level of reduced GSH in these cells is shown in Table 3. Concentration of GSH in normal lymphocytes showed a range between 3.67 and 6.44 μmol in 10^6 cells with an average of $5.43 \pm 0.48 \mu\text{mol}$. This GSH concentration was depleted by 84% of the control value after 5 h treatment with BSO. The statistical difference between the mean GSH concentrations of these two groups was significant.

Western blot analysis

Representative results of the Western blot analysis are illustrated in Fig. 1. β -Actin was included as an internal indicator in all analyses to control for potential discrepancy in sample loading. The level of p53 protein was extremely low in normal PHA-unstimulated cells; it was raised after 5 h and significantly more so after 10 h of PHA stimulation. In unstimulated lymphocytes, very little enhancement was observed in the level of p53-protein after irradiation with or without BSO or GSH. However, in PHA-stimulated cells the level of p53 protein was increased considerably after irradiation. With respect to radiation alone, the level of p53 protein in G₁ cells was increased significantly in BSO +4Gy cells, whereas the level was much lower in GSH +4Gy cells. The level of p21 protein was increased significantly in both stimulated and unstimulated lymphocytes after irradiation; it was higher in BSO +4Gy sample and less in GSH-pre-treated cells than in PHA-stimulated samples with radiation alone.

Table 3. Levels of GSH in HPBLs 5 h after a single treatment of BSO

Sample no.	BSO (mM)	Total GSH ($\mu\text{mol}/10^6$ cells)	Mean $\pm$ SEM (reduction%)
1	0	3.67	5.43 ± 0.48
2	0	6.44	
3	0	5.57	
4	0	5.04	
5	0	6.41	
6	5	0.85	0.86 ± 0.05^a (−84%)
7	5	0.78	
8	5	0.98	
9	5	0.79	
10	5	0.92	

^a $P < 0.05$ a student's *t*-test.

Figure 1. Immunoblotting analysis of X-ray-induced p53 and p21 protein expression in human lymphocytes irradiated before and after PHA stimulation. Lymphocytes were exposed to 3.5Gy X-rays (a) at G_0 stage before PHA stimulation, and kept at 37 °C for 6 h (b) at G_0 stage before PHA stimulation, and kept at 37 °C for 1 h before PHA addition for 5 h and (c) at G_1 stage, 10 h after PHA stimulation, and kept at 37 °C for 6 h. Proteins were extracted 6 h after irradiation.

DISCUSSION

Data here show that X-irradiation delays lymphocytes in their passage through the cell cycle. The extent of delay was more in 72-h fixed samples than in 52-h fixed ones and a similar trend was observed in the irradiated cells where a higher level of delay was induced in 65/70 h than in 44/48 h fixed cells (Purrott *et al.* 1980; Hoffmann *et al.* 2002). It has been suggested that first-division lymphocytes fixed for different time periods show similar radiosensitivity (determined by CAs induction) (Scott & Lyons 1979), whereas differences were reported in other studies (Bender & Brewen 1969; Beek & Obe 1976). By using the fluorescence *in situ* hybridization technique, it was observed that late-arising metaphases are more aberrant than early-arising ones (Boei *et al.* 1996; Hoffmann *et al.* 2002). By classical cytogenetic analysis, the present study has shown more aberrations in 72 h fixed samples than in those at 52 h. Therefore, possibly late-arising metaphases are more sensitive to radiation than early arising ones and have shown more delay in cell cycle progression.

The novel aspect of the present study is the analysis of the influence of endogenous GSH level on radiation-induced delay through the cell cycle. Exogenous addition of GSH reduced radiation-induced micronuclei (Mazur 2000) and CAs (Chattopadhyay *et al.* 1999) in mammalian cells. However, reports on the influence of GSH on radiation-induced delay in cell kinetics are scanty. Here, concentration of GSH was selected on the basis of our earlier study where 15 mM GSH showed better protection against radiation-induced CAs and delay in cell cycle progression than 10 mM and 25 mM concentrations (Chatterjee & Jacob-Raman 1986). In the present study, addition of exogenous GSH removed the radiation-induced delay more convincingly in early first-division cells than late first-division cells. It could be that this delay-reducing effect of GSH was not corresponding to the reduction in the frequency of CAs, as GSH pre-treatment reduced the radiation-induced CAs significantly in late-arising metaphases irradiated in the G_0 stage but failed to reduce delay in the course of the cell cycle. It has also been observed that there are cell membrane and cytoplasmic proteins that regulate lymphocyte proliferation

(Noelle & Lawrence 1981). Oxidation of cell surface thiol groups in lymphocytes can ablate the mitogen-induced proliferative response, and exogenous supply of GSH can reverse this by scavenging free radicals (Pahlavani & Harris 1998). Therefore, it seems that in the present study, free radicals and reactive oxygen species (ROS) generated after irradiation might have inhibited the mitogen-induced proliferative response of the cells by altering the cell membrane and cytoplasmic protein thiol groups and also by inducing DNA damage. Presence of GSH might reduce the damage to these targets by scavenging free radicals and thus protect the cells.

In this investigation, it was observed that the degree of protection of CAs by GSH-pre-treatment was lower in G_1 -irradiated cells than in G_0 -irradiated cells. It is known that unstimulated G_0 -lymphocytes do not synthesize DNA and show lower physiological and metabolic activity than stimulated lymphocytes (Kay *et al.* 1975; Torelli *et al.* 1981). Therefore, it is likely that the consequences of GSH-pre-treatment to G_1 cells would be different from those to unstimulated G_0 -cells and thus would show poor protection of irradiation-induced CAs in G_1 cells and even fail to reduce irradiation-induced delay in cell cycle progression in the late first-division cells.

Present data indicate that the depletion of GSH by BSO did not affect the pattern of delay induced by irradiation; however, there was an increase in the frequency of chromosomal aberrations. The reduced-GSH estimation in this study indicates that 5 h incubation with BSO (5 mM) could deplete the GSH level significantly with respect to controls. In cultured cells, more than 75% depletion was achieved within 4–5 h duration by 500 μ M to 10 mM of BSO (Shrivie *et al.* 1985; Edgren & Revesz 1987). It has been shown that such depletion of endogenous GSH by BSO increases the frequency of CAs induced by arecoline (Deb & Chatterjee 1998) and mitomycin C (Dev-Giri & Chatterjee 1998). It has been shown that BSO-mediated GSH depletion increases radiation-induced CAs, except in exchange aberrations, in human lymphocytes (Chattopadhyay *et al.* 1999) this could be due to reduction in the DNA shielding effect of GSH, and which would enhance DNA strand break induction, probably by hydroxyl radicals, because increase in damage by removing this natural protection system is largely due to the effect of hydroxyl radicals (Nygren *et al.* 1995). In this study BSO-treatment enhanced the frequency of CAs almost uniformly at all stages of the cell cycle; nevertheless, the extent of radiation-induced mean delay was not enhanced, rather it marginally reduced in BSO-treated G_0 cells. There are reports that BSO can scavenge irradiation-derived free radicals and affords protection of dry barley seeds against irradiation (Singh & Kesavan 1993) and athymic mice carrying tumour xenografts (Halperin *et al.* 1992). Accordingly in the present study, a few molecules of BSO, either remaining in the solution or in the cytoplasm during irradiation, might possibly protect cell membranes from irradiation damage. Such BSO-mediated protection could be the reason for the lack of increase in the irradiation-induced delay in the course of the cell cycle, in spite of increased frequency of CAs.

Bender and Brewen (1969) proposed the existence of two subpopulations of PHA-stimulated lymphocytes that differ in radiosensitivity and in the rate of progression from PHA stimulation through cell division. It may be possible that late-arising first divisions are delayed in their responsiveness to PHA or progression through the S and G_2 stages to mitosis, and if these cells should be aberrant, then their responsiveness to PHA would be delayed further. Such a mitotic lag in damaged cells is a commonly observed phenomenon, which has suggested that heterogeneity in numbers of divisions in culture can be explained by the time required for the first DNA synthesis after PHA stimulation (Morimoto & Wolff 1980). It has been demonstrated that cells can remain arrested in the G_1 phase of the cell cycle following DNA damage caused by ionizing radiation in order to repair or recover from the induction of DNA lesions (Rudoltz *et al.* 1996). It is well known that nuclear phosphoprotein encoded by the tumour

suppressor gene, *p53* is a crucial component of the cellular pathways that are invoked in response to DNA damage. Several studies have demonstrated that *p53* regulates the G_1 checkpoint (Kuerbitz *et al.* 1992) through the transcriptional up-regulation of the cyclin-dependent kinase inhibitor *p21/Waf1/Cip1* (Harper *et al.* 1993). Here, the level of *p53* protein after irradiation was raised marginally in PHA-unstimulated cells and significantly so in PHA-stimulated cells. However, the level of *p21* was enhanced significantly in both PHA-unstimulated and -stimulated cells. It has been demonstrated that there was a reduction in *p53* response in quiescent compared to PHA-stimulated cells after irradiation (Fukao *et al.* 1999). It was suggested that the level of ataxia-telangiectasis mutated (ATM), which is extremely low in unstimulated lymphocytes, was increased markedly in mitogen-stimulated lymphocytes (Fukao *et al.* 1999). ATM is a key initiating factor in the cascade of events leading to activation of DNA damage, responsive signalling pathways and cell cycle checkpoints (Savitsky *et al.* 1995). One of the important targets for ATM is *p53*. Therefore, such low levels of ATM in the unstimulated lymphocytes could be a factor in the lack of induction of *p53* protein after irradiation in this study.

Expression of *p21* has been shown to be regulated largely at the transcriptional level by both *p53*-dependent and -independent mechanisms (Gartel & Tyner 2002). The present data indicate that significant increase in *p21* in unstimulated G_0 lymphocytes could be a factor responsible for irradiation-induced *p53*-independent cell cycle arrest. The presence of GSH reduced the level of *p21* with respect to irradiation alone. In fact, irradiation of GSH-pre-treated cells reduced the level of both *p53* and *p21* proteins. This observation corresponds well with the low frequency of CAs. Such reduced levels of *p53* and *p21* could also be the additional factor for GSH-mediated reduction in irradiation-induced delay in the cell cycle, besides protection of cell membrane and cytoplasmic proteins. On the other hand, irradiation to BSO-treated cells increased the frequency of CAs, showed higher levels of *p53* and *p21* proteins and maintained the delay in the cell cycle induced by irradiation. However, the present Western blot analysis does not provide information concerning levels of *p53* and *p21* in early and late-arising first-division cells, although it does provide an indication of the expression of these proteins, which regulate the cell cycle plus repair/recovery events after irradiation.

Therefore, it may be inferred from the results shown previously that the targets for irradiation-induced delay in lymphocyte proliferation and induction of CAs are not the same but different and that the presence of GSH reduces the free radicals and protects these targets from radiation. Depletion of endogenous GSH by BSO increases the levels of free radical-induced CAs without further increase in the delay of cell cycle. However, irradiation up-regulates and stabilizes nuclear *p53* protein and leads to kinetic arrest of the cells, through transcriptional recruitment of *p21*. Thus, it seems that two components contribute to the induction of delay in progression of the cell cycle after irradiation. These are (1) the cell membrane-sulfhydryl (SH) status and cytoplasmic proteins that regulate lymphocyte proliferation are determined, and (2) the DNA damage checkpoint that communicates information between a DNA lesion and components of the cell cycle. The latter triggers *p53/p21*-mediated cell cycle arrest. It may be further suggested that an increase in the level of endogenous GSH would act directly on the first component and indirectly on the second by reducing the frequency of CAs. However, the reason behind the poor ability of GSH to reduce irradiation-induced delay in the cell cycle of the late-arising first-division cells, in spite of reduction in the frequency of CAs (G_0 -cells at 72 h), could be the result of their inherent property of delayed response to PHA.

The present study on the influence of endogenous GSH status in irradiated cells provides information in understanding the mechanisms of protection of endogenous GSH on irradiation-induced delay in cell cycle.

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