Trakia Journal of Sciences, Vol. 3, No. 1, pp 37-42, 2005

Available online at:
http://www.uni-sz.bg

ISSN 1312-1723

Original Contribution

INFLUENCE OF CONDITIONING ON CELL SURVIVAL AND INITIAL CHROMOSOME DAMAGE IN X-IRRADIATED HUMAN CELLS

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ABSTRACT

Purpose: The aim of this study was to investigate the influence of a conditioning dose of 0.1 Gy on cell survival and initial chromosome damage in human cells.
Material and methods: Human lymphocytes and primary fibroblasts were used in this study. Cells were exposed to 0.1 Gy, followed by 1, 2, 4 and 6 Gy of X-irradiation. Clonogenic survival experiments were done during the 4 and 24 hours time intervals that occurred between the conditioning and the challenging doses. The frequency of initial chromosome damage was assessed using premature chromosome condensation assay. The conditioning dose was given 4 hours prior to the challenging doses of 4 and 6 Gy.
Results: The conditioning dose of 0.1 Gy did not improve the cell survival levels significantly in primary human fibroblasts. The time of conditioning of either 4 or 24 hours did not have any effect on the cell survival curve. Premature chromosome condensation technique applied after 4 and 6 Gy challenging doses showed a significant difference between induced frequency of breaks in conditioned and unconditioned lymphocytes.
Conclusion: Conditioning dose of 0.1 Gy has an influence on initial chromosome break formation but not on cell survival.

Key words: adaptive response, low doses, premature chromosome condensation, cell survival

INTRODUCTION

It is widely known that different types of DNA lesions are induced as a result of interaction between ionising radiation (IR) and the cellular DNA via both direct and indirect action. These include single- and double strand breaks (SSB and DSB), abasic sites, DNA-DNA and DNA-protein crosslinks, together with modifications of nucleobases The cells may cope with DNA damage through several repair processes and activation of the cell cycle checkpoints. DNA DSB is found to be the major lesion responsible for the formation of chromosomal alterations. DNA DSB can be repaired through two different repair processes, non-homologous end-joining and homologous recombination. Unrepaired and misrepaired DNA lesions lead to mutations, chromosome aberrations, cell transformation and cell death.

High doses of IR produce clinically detectable damage on living organisms and the quantification of low doses radiation are not simple. At the same time, low doses of radiation may likely activate mechanisms of increased resistance by which irradiated cells become radio-resistant to higher doses.

For example, it has been demonstrated that in vitro pre-treatment of cell systems with low doses of X-ray leads to a response that makes these cells less sensitive to the effects of subsequent irradiation to a higher dose. This phenomenon called, Adaptive Response, (AR) was first observed in lymphocytes by Oliviery et al. (1). They reported that the frequency of chromatid aberrations was decreased by 50% in the adapted cells compared to the expected number after exposure to 1.5 Gy of X-rays. Such an AR has been observed for several end-points including chromosome aberrations, chromatid aberrations, sister chromatid exchanges, micronucleus formations (2, 3, 4, 5), mutations (4), DNA strand breaks (6) and cell

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survival (7).

It is generally accepted that AR is related to a reduction of damage by induction of radical detoxification (8) and repair pathways (6, 9).

It is important to state that the adaptive treatment does not concomitantly create the expression of AR. There are reports indicating lack of radio-adaptive responses in cultured human lymphocytes (10). AR was reported for m5S cells in terms of cell survival whereas HeLa cells did not show adaptation (7). Mutagenic adaptation coupled with survival adaptation has been observed in some studies (8) but not in others (4, 11). It was shown that morphological transformation in adapted mouse cells increased, whereas these cells were more resistant to the gene mutation induction, clastogenic damage and cell killing (12).

In the work described here we studied the influence of conditioning dose of 0.1 Gy on chromosomal breaks formation and cell survival. Confluent human fibroblasts and Go (non-stimulated) lymphocytes were used for experiments in order to avoid the possible role of the cell cycle distribution on AR. The cells were treated with the conditioning dose 4 hours before exposure to challenging doses for chromosome damage experiments and both 4 and 24 hours time before the challenging dose at clonogenic survival experiments. Initial chromosome break formation was detected by premature chromosome condensation method (PCC). The PCC assay makes it possible to study the initial damage of chromosomes shortly after the irradiation (13, 14) and it does not require cell-culturing time. It is a simple and sensitive method. No reports on using of PCC assay in AR studies have been found so far.

METHODS

Cell culture

Human primary fibroblasts VH16 were grown in Dulbecco's modified Eagle Medium (DMEM) and Ham's F10 medium at 37ºC. The media were supplemented with 15% foetal bovine serum (FBS); penicillin and streptomycin antibiotics were present. The culture was aerated by 5% CO_2 atmosphere with 95% humidification. Cells were split 1:2 weekly. All experiments were performed with cells grown to confluence.

Human peripheral blood lymphocytes isolated by Ficoll-Hypaque gradient system, frozen and kept in liquid nitrogen were thawed in Rosewell Park Memorial Institute (RPMI) medium, supplemented with 40% FBS. The mononuclear lymphocytes were kept overnight at 37°C in a humidified atmosphere of 5% CO_2 air mixture.

Irradiation

Confluent fibroblasts and G0 lymphocytes were irradiated with an Andrex X ray-machine at 200 kV and 4 mA. Low dose of 0.1 Gy was applied at dose rate of 0.2 Gy/min. Challenging doses of 1, 2, 4 and 6 Gy were applied at a dose rate of 1.8-2 Gy/min.

Determination of plating efficiency

For survival fraction experiments the challenging dose was applied 4 and 24 hours respectively after exposure to a conditioning dose of 0.1 Gy. After the challenging dose cells were trypsinised and counted on a coulter-counter ZM. 500 cells per dish were seeded. 5 dishes were used for each radiation dose point. After seeding the cells were placed in the incubator for 2 weeks. The medium in each dish was carefully refreshed once. After two weeks the dishes were removed from the incubator. The medium was discarded and colonies were fixed in 5 ml 0.9% NaCl for a few seconds. After aspirating away the fixing solution the dishes were dried in an oven (for 2 hours) then filled with methylene blue (2.5 g/l) for a few minutes, rinsed with water two times and dried again. Stained dishes were counted for colonies by the naked eye. All visible colonies were counted.

The plating efficiency (PE) for the non-irradiated cells was calculated by the following formula: PE= (colonies counted/cells seeded) x100%.

The survival fraction (SF) of irradiated cells was calculated by the following formula: SF= [colonies counted/(cells seeded x PE)] x100%.

Premature chromosome condensation (PCC)

Human lymphocytes isolated by Ficoll-Hypaque gradient system and X-irradiated were fused with mitotic Chinese hamster ovary (CHO) cells immediately after the irradiation. The protocol used was modified to increase the yields of PCCs (14). 150 µl 40% polyethylene glycol (PEG) prepared and kept on ice before fusion of the cells was added into each tube directly to the cell pellet. 3 ml Ham's F10 medium was added very slowly, 0.5 ml six times within 3-4 min. Cells were centrifuged for 5 min at 900 rpm. After the centrifugation and discarding the supernatant

750 µl of Ham's F10 medium supplemented with 40% FBS and 30 µl colcemid (1 µg/ml) was added. The cells were incubated at 37°C for 1 hour. After PCC induction, fixation was performed. 8 ml hypotonic solution (KCl, 0.075 M) was added to each tube. Then the cell suspension was transferred into a conical tube and incubated for 20 min. at 37 °C. Cells were fixed in 5 ml fixative solution (methanol:acetic acid, 4:1). The cells were dropped with a drawn-out Pasteur pipette on clean slides and stained with 2% Giemsa for 4 min. Microscopic examination was performed with a Zeiss light microscope using 100x objective. The number of chromosome fragments was counted in at least 70 cells for each dose point.

RESULTS

Survival curves for fibroblasts after X-ray irradiation

In **Figure 1a** and **1b** survival curves for conditioned and non-conditioned cells are shown.

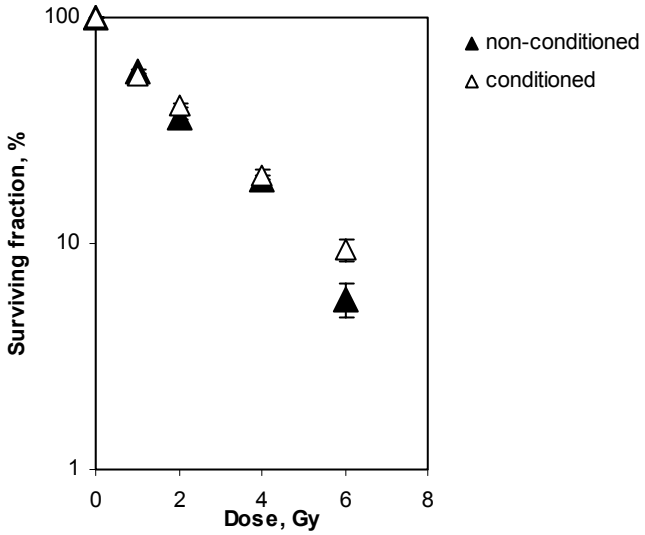

Figure 1a: *"Survival curves of cells conditioned with 0.1Gy 4 hours before challenging dose and of non-conditioned cells. Bars represent standard deviation."*

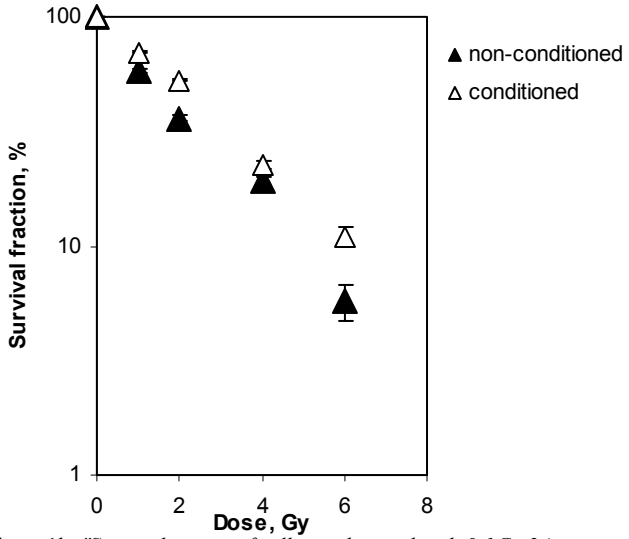

Figure 1b: *"Survival curves of cells conditioned with 0.1Gy 24 hours before challenging dose and of non-conditioned cells. Bars represent standard deviation."*

In order to determine cell survival, the cells were exposed to challenging doses of 1, 2, 4, and 6 Gy. To test whether a conditioning dose of 0.1 Gy influences radiosensitivity, cells were treated with the low dose of 0.1 Gy, 4 and 24 hours before the challenging dose. In **Figures 1a** and **1b** survival fraction of non-irradiated cells as well as the survival fraction of conditioned unchallenged cells is set at 100%. The plating efficiency of non-irradiated cells is 21.68. No significant shift in the survival curve is observed for cells

conditioned 4 and 24 hours, respectively, before the challenging dose (p=0.9 and p=0.7). On **Table 1** the dose to reduce the surviving fraction to 37% (D_{37}) is given.

Table 1: *Doses required to decrease surviving fraction to 37% for conditioned and non-conditioned cells*

Treatment	D_{37} (Gy)
Non-conditioned	2.0
Conditioned 4h	2.4
Conditioned 24h	2.6

As it is shown on **Table 1** there is a difference between D_{37} of cells conditioned 4 and 24 hours before the challenging dose and D_{37} of non-conditioned cells. The D_{37} is higher in the conditioned cells compared to the non-pre-treated cells but the difference is not significant.

Assessment of initial DNA damage induction in prematurely condensed human lymphocytes.

Frequency of induced breaks per cell in prematurely condensed chromosomes in conditioned and non-conditioned cells was determined. The repeated experiments for two different donors A and B are presented on **Tables 2** and **3**, respectively.

Table 2*: Frequency of breaks per cell in conditioned and non-conditioned cells examined by PCC for donor A*

Donor	**Dose/Gy**	**Number of cells scored**	**Frequency of PCC/cell**	**Induced frequency of Breaks/cell**
A	0	71	46	0
	0.1+0	126	46.1	0.1
	4	150	63.3	17.3
	0.1+4	150	61.4	15.4
	0.1+0	124	46.2	0.2
	6	150	75.1	29.1
	0.1+6	150	71.1	25.1

Table 3: *Frequency of breaks per cell in conditioned and non-conditioned cells examined by PCC for donor B*

Donor	**Dose/Gy**	**Number of cells scored**	**Frequency of PCC/cell**	**Induced frequency of Breaks/cell**
B	0	70	46	0
	0.1+0	27	46.2	0.2
	4	80	62.9	16.9
	0.1+4	82	61.6	15.6
	0.1+0	82	46.2	0.2
	6	110	75.3	29.3
	0.1+6	98	71.3	25.3

In order to measure initial damage at chromosome level (breaks), lymphocytes from two different donors were fused with CHO cells immediately after the challenging doses of 4 and 6 Gy. To test whether a conditioning dose has an influence on the induction of PCCs, cells were exposed to 0.1 Gy 4 hours before the challenging dose. As demonstrated on **Tables 2** and **3**, no excess of breaks was found in the unexposed group. In both experiments exposure of lymphocytes to a single dose of 0.1 Gy increased the frequency of PCC in comparison to non-irradiated samples. PCC induction of cells exposed to the 0.1 Gy prior to the challenging dose of 4 Gy and 6 Gy is significantly lower compared to cells exposed to challenging dose alone ($p<0.05$).

From pooled data for 4 Gy the mean difference from the two donors is 1.8 breaks per cell, and for 6 Gy it is 4.2. These data reveal occurrence of an adaptive response for initial frequency of DNA damage as determined using PCCs assay.

DISCUSSION

There are large amount of data in the scientific literature on testing various conditioning doses (1 cGy to 1 Gy) for their ability to induce AR, different biological end-points examined in different cell systems. In the present study the influence of conditioning dose of 0.1 Gy on cell survival and

chromosome breakage in confluent fibroblasts and Go lymphocytes was studied.

Pre-treatment of normal human fibroblasts with conditioning dose of 0.1 Gy 4 and 24 hours, respectively, before the challenging dose had no significant effect on clonogenic survival compared with cells directly irradiated with the challenging doses. The difference between D_{37} values of non-conditioned cells and D_{37} of conditioned cells 4 and 24 hours, respectively, are not significant. The time of conditioning of 4 or 24 hours does not have any effect on cell survival curve. The data found in the literature in terms of AR studies on cell survival end point are controversial. Various systems have been tested for AR induced by small doses of radiation using survival end-point showing the significant influence of conditioning on survival fraction. Survival adaptation was observed for confluent mouse embryonic skin M5-S cells exposed to 0.02 Gy 5 hours before challenging dose of 3 Gy (7). Significant increase in cell survival was observed in plateau–phase CHO cultures pre-treated with Xanthine-Xanthine oxidase (X-Xo) and challenged with gamma irradiation (500-1500 rad) 24 hours after priming (8). Besides, there are studies indicating lack of improvement of the cell survival. No improved clonogenic survival levels were detected in confluent C3H10T1/2 mouse embryo cells treated with adapting dose of 0.1 to 1.5 Gy 3.5 hours before 4 Gy acute challenging dose (4). Confluent normal fibroblasts priming with IR (0.01 to 50cGy) then challenged with higher IR did not demonstrate increased survival (15). The different observations from different studies indicate that AR cannot be induced in all cell systems and it shows its complex mechanism and variation in different type of cells.

A significant difference was found between induced frequency of breaks in conditioned and unconditioned lymphocytes for 4 and 6 Gy challenging doses for both donors using premature chromosome condensation technique. This implicates occurrence of an adaptive response for the initial frequency of observed DNA damage. The PCC technique allows visualisation of initial chromosome breaks immediately after the challenging dose before the probable induced repair takes place. So, the noticed adaptive response could be due to increased oxidative defence processes as well as activated processes at molecular level involved in AR mechanisms.

AR phenomenon was noticed for initial frequency of chromosomal aberrations using PCC technique. Slight shift of survival curve is observed for conditioned fibroblasts, which is not statistically significant.

From our results it would appear that conditioning of 0.1 Gy has an influence on radiation-induced initial chromosome breaks but not on clonogenic survival.

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