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Radio-adaptive response of peripheral blood lymphocytes following bystander effects induced by preirradiated CHO-K1 cells using the micronucleus assay

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► ***Original article***

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ABSTRACT

Background: Radio-adaptive response and bystander effects are known phenomena occurring in cells following exposure to ionizing radiation (IR). In this study we examined possible radio-adaptation of lymphocytes following bystander effects induced by CHO-K1 cells. ***Materials and Methods***: Whole blood and CHO-K1 cells were cultured in RPMI-1640 complete medium. Cells were separately irradiated with various doses of gamma rays. A co-culture was set to examine the bystander effects induced by CHO-K1 cells on lymphocytes exposed later to a challenge dose of 4 Gy. Treated cells were exposed to cytochalasin-B to arrest cells in cytokinesis stage. Micronucleus (MN) as end point was scored in binucleate cells after staining in Giemsa. ***Results***: The frequency of MN increased significantly with increasing dose of radiation both in lymphocytes and CHO-K1 cells ($p<0.001$). Although, no significant difference was observed between control non-irradiated cells and those exposed to 0.2 Gy ($p>0.05$). Co-culture of the non-irradiated lymphocytes with pre-irradiated CHO-K1 cells significantly reduced the mean frequency of MN in lymphocytes irradiated with a dose of 4Gy ($p<0.001$). ***Conclusion***: Results showed that bystander effects induced by gamma-irradiated CHO-K1 cells led to induction of radio-adaptive response in lymphocytes. The mechanism by which radio-adaptive response is induced following bystander effect is not clearly known, however cellular signaling and genome instability induced in cells indirectly might be considered as possible triggering events for radio-adaptive response.

Keywords: *Bystander effect, radio-adaptive response, gamma rays, micronucleus assay.*

INTRODUCTION

Biological effects of low-dose radiation have attracted much interest since last two decades [1]. According to the target theory, cellular damage occurs only during or a short period after the energy deposition in cellular bio-molecules directly or indirectly via free-radicals production [2, 3]. However, numerous scientific research papers show that low dose irradiation is stimulatory and/or beneficial in a wide variety of microbes, plants, invertebrates, and vertebrates [4, 5]. These evidences show that radiation has a stimulating effect on a number of biological processes and can induce resistance against higher doses of ionizing radiation a phenomenon known as

radiation hormesis, also known as adaptive response. During the past three decades, considerable interest has been focused on inducible cellular processes occurring in cells following exposure to low doses of ionizing radiation. Similar to all biological responses of cells to IR induced or exogenous stress, this process is also mediated through DNA damage [6, 7]. There are now evidences showing that irradiated cells can influence on neighboring non-irradiated cells through signaling events causing bystander effects. These signaling events might be mediated by the reactions of radiation induced free radicals on DNA, with the existence of a threshold at which the bystander signal is not operative (0.1 Gy dose of X-rays) [8].

Radiation-induced bystander effects are responses, normally associated with directly irradiated cells, observed in non-irradiated cells as a result of receiving signals from irradiated cells [9, 10]. There is evidence that bystander signals can induce genomic instability both *in vivo* [11, 12] and *in vitro* [13, 14]. On the other hand, the implication of oxidative processes in bystander effects has been shown in many indirect experiments using radical scavengers or antioxidants [15-17].

If radio-adaptive response induced by low doses of ionizing radiation in hit cells occur through stimulating effects of oxidative stress or factors involved in DNA damage, then it may be expected that bystander induced in non-hit cells might also induce a type of radio-adaptive response.

Radio-adaptive bystander effects were also induced in non-irradiated cells by a transmissible factor (s) present in the medium of cells exposed to different doses of γ-radiation. This radio-adaptive bystander effect was correlated with a reduced cellular level of protein p53 and an increase of intracellular reactive oxygen species and enzymes involved in DNA repair [18].

Therefore the aim of this study was to investigate possible bystander effects in human lymphocytes induced by irradiated CHO-K1 cell line, and radio-adaptive response of bystander lymphocytes.

MATERIALA AND METHODS

Blood samples

Blood samples were obtained repeatedly from a 35 years old male volunteer with his consent during the study. He was not a cigarette smoker and was not exposed to ionizing radiation and antibiotics and had no history of infectious diseases for 3 months prior to sampling. This study was approved by the Ethical committee of Shahid Beheshti University of Medical Sciences (Tehran, Iran).

Cell culture and slide preparation

Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation was used to purify peripheral blood mononuclear cells (PBMNC). PBMNC were distributed in the Eppendorf micro -tubes containing PRMI-1640 medium (Gibco-BRL). CHO-K1 cells (Kept in N_2 freezer at Novin Medical Radiation Institute) were cultured in RPMI 1640 medium supplemented with 10% heat inactivated Fetal Calf Serum (FCS) (Gibco-BRL), antibiotics (Penicillin 100 iu/ml and streptomycin 100 µg/ml) (Gibco-BRL) and incubated at 37°C in an atmosphere containing 5% CO_2 in air. Samples were exposed to various doses of gamma-rays (0.2, 2 and 4Gy) generated by a therapeutic ^{60}Co source (Theratron II, 780 C, Canada), at a distance from source to sample of 80 cm, and a dose-rate of 57.1cGy/min in room temperature (23 ± 2 37° C). A control non-irradiated group was also considered for the study. Control and irradiated CHO-K1 cells were harvested 28 hours after cytochalasin B (Sigma–Alderich, 5 mg/ml final concentration) treatment by gentle trypsinization (0.25% trypsin-EDTA, Gibco-BRL) for 5-10 min at 37°C . After washing cells in hypotonic solution (KCl, 0.075M), cells were fixed in Carnoy's fixative (Methanol : Acetic Acid, 3:1 v/v). Then slides were prepared and stained in Giemsa. Control and irradiated PBMNC samples were also cultured in RPMI-1640 supplemented with 10% Fetal Calf Serum (FCS) (Gibco-BRL), antibiotics (Penicillin 100 iu/ml and Streptomycin 100 µg/ml) (Gibco-BRL) and 0.1 ml phytoheamaglutinin (PHA) (Gibco-BRL),

then incubated for 44 hours at 37 °C. Twenty eight hours before harvesting, cytochalasin B (Sigma-Aldrich) was added to the cultures at a final concentration of 5µg/ml. Harvesting and slide preparation was done according to the standard protocols [19]. Slides were stained in Giemsa 10% for 10 min. Thousand binucleate cells were scored for each sample two sides blinded with a light microscope (×400 magnification) according to the described criteria [20].

Co-culture of lymphocytes with CHO-K1 cells

CHO-K1 cells were exposed to doses of 0.2 Gy and 2 Gy of gamma rays as described earlier. One hour later non-irradiated PBMNC were added to the culture vessels. According to Yang *et al.* (2007) the maximum percentage of bystander signaling occurs as early as 1 hour after irradiation [21, 22]. PBMNC were co-cultured with CHO cells for a period of 18-20 hours. Then, PBMNC were carefully separated from adherent CHO-K1 cells and centrifuged for 10 min at 2000 rpm. Aliquots of PBMNC were then irradiation with a dose of 4Gy as a challenging dose with aforementioned irradiation condition. After irradiation, PBMNC culturing, harvesting, slide preparing, staining and cell counting was similar to that cultures irradiated with various doses of gamma rays. At least, 2000 binucleate cells were scored for each sample two sides blinded. Figure 1 shows examples of binucleate cells with or without micronuclei.

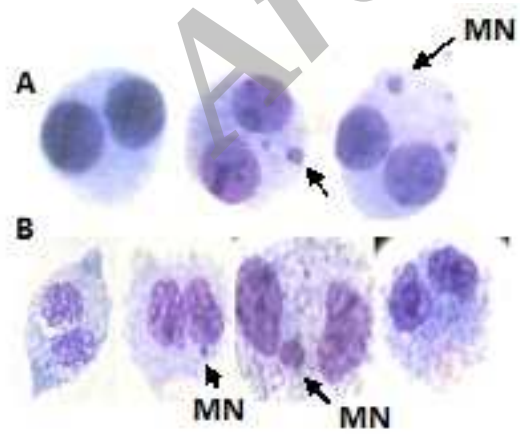

Figure 1. A) Binucleate lymphocytes arrested at cytokinesis stage of the cell cycle without and with micronuclei (MN); B) Binucleate CHO-K1 cells with or without micronuclei (MN), stained in Giemsa. Magnification ×400.

Statistical analysis

For data statistical analysis SPSS software (version 16.0) was used and statistical analysis was performed using non-parametric analysis of variance (ANOVA) and non-parametric Mann-Whitney *U-test*. P value less than 0.05 was considered as significant.

RESULTS AND DISCUSSION

Results are summarized in table 1 and shown in figures 2 and 3. As seen, the mean frequency of micronuclei increased significantly in CHO-K1and lymphocytes after irradiation compared with the control group ($p<0.001$) (table 1).

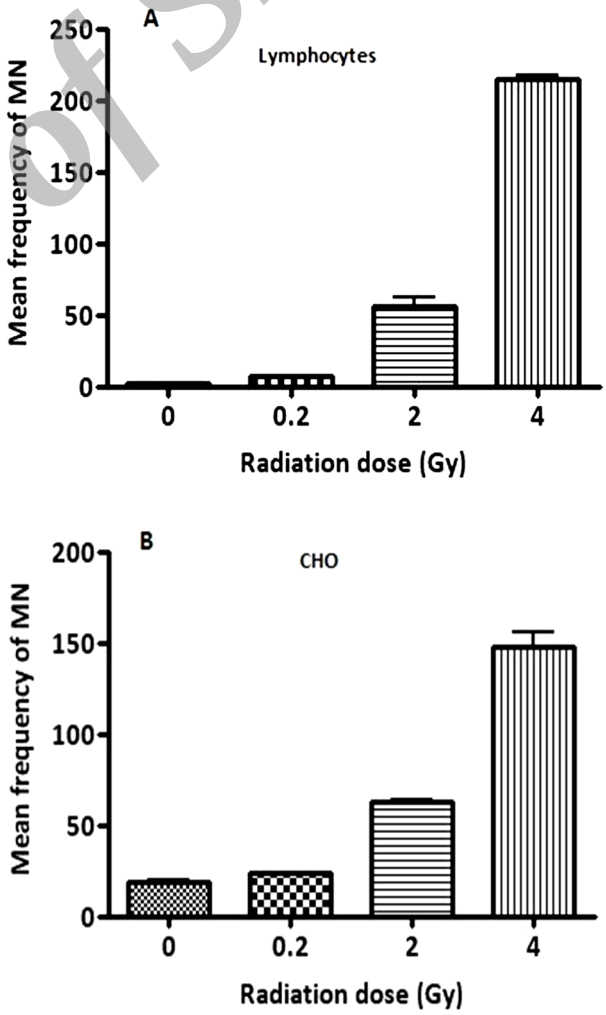

Figure 2. Mean frequency of micronuclei induced by various doses of gamma rays. A: Lymphocytes. B: CHO-K1 cells.

Table 1. Summary of data obtained for various treatment groups.

Cells studied	Radiation dose (Gy)	No. of Binucleate analyzed	Total number of micronuclei observed	Statistical significances
Lymphocytes	0	1000	5	
	0.2	1000	13	p>0.05
	2	1000	112	P<0.001
	4	1000	428	P<0.001
CHO-K1	0	1000	38	
	0.2	1000	47	p>0.05
	2	1000	125	P<0.01
	4	1000	296	P<0.001
Lymphocytes + CHO-K1	0 + 0	1000	5	p>0.05
	0 + 0.2	1000	3	p>0.05
	0 + 2	1000	6	p>0.05
Lymphocytes after co-culture with CHO-K1 (0.2 Gy)	4	2000	119	P<0.01*
Lymphocytes after co-culture with CHO-K1 (2 Gy)	4	2000	219	P<0.01*

*Compared with lymphocytes irradiated with 4 Gy gamma rays alone.

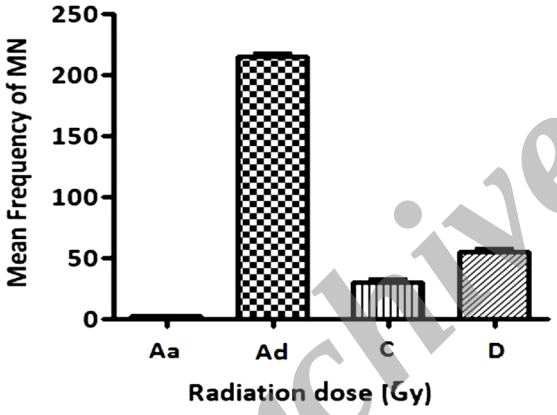

Figure 3. Mean frequencies of micronuclei in lymphocytes observed in different treatment groups. Aa: Non-irradiated lymphocytes; Ad: Lymphocytes irradiated with 4Gy gamma rays; C: Bystander lymphocytes with 0.2Gy irradiated CHO cells irradiated with 4Gy gamma rays; D: Bystander lymphocytes with 2Gy irradiated CHO cells irradiated with 4Gy gamma rays.

The mean frequency of micronuclei decreased remarkably up to seven folds in lymphocytes exposed to 4 Gy gamma rays following co-culture of non-irradiated lymphocytes with CHO-K1 cells pre-irradiated with a dose of 0.2 Gray ($p<0.001$). Similarly a 4 fold decrease in the frequency of micronuclei was observed in lymphocytes exposed to a dose of 4 Gy following co-culture with CHO-K1 cells exposed to 2 Gy gamma rays ($p<0.001$) (figure 3 and table 1).

Radiation-induced bystander effects are responses, normally associated with directly irradiated cells, observed in non-irradiated cells as a result of receiving signals from irradiated cells [9, 10]. These signaling events might be mediated by the reactions of radiation induced free radicals on DNA [8].

The production of the bystander signal leading to an increase of frequency of micronuclei in non-targeted cells is to some extent independent of the level of DNA damage in the irradiated cells which produce the signal [16]. Therefore it is evident that co-culture of lymphocytes with irradiated CHO-K1 cells in this study may induce some kind of bystander effects in lymphocytes.

There is a bulk of evidence that radiation has a stimulating effect on a number of biological processes and can induce resistance against higher doses of ionizing radiation [23]. Adaptive response is one of the most attractive responses

of cells to low dose ionizing radiation. The adaptive response, first reported by Samson and Carins (1977) [24] in Escherichia coli has also been observed in some higher mammalian systems [25] and in lymphocytes exposed to low dose of ionizing radiation [26, 27]. Most of these studies were performed in *in-vitro* conditions and now, there is little doubt that the *in-vitro* pretreatment of human lymphocytes with low doses of X-rays makes these cells less susceptible to cytogenetic damage by subsequent high acute doses of X- or gamma -rays [28-30]. This adaptive response to IR, occurs after very low exposures (0.005–0.01 Gy) which do not induce observable cytogenetic damages by themselves [31]. In this study the frequency of micronuclei observed for lymphocytes co-cultured with irradiated CHO-K1 was lower of similar to that observed with non-irradiated CHO-K1 cells (table 1). However, when these cells were irradiated with higher dose of gamma rays (4 Gy) a considerably significant reduction in the frequency of micronuclei was observed (Figure 3). This reduction might be attributed to the bystander effect induced in lymphocytes following co-culture with irradiated CHO-K1 cells.

It was shown that bystander effect can influence on DNA repair pathways to alter cellular response from a hyper radio-sensitivity state to radio-resistance [32, 33]. Radio-adaptive bystander effects were also induced in non-irradiated cells by a transmissible factor(s) present in the medium of cells exposed to different doses of γ-radiation. This radio-adaptive bystander effect might be correlated with an increase of intracellular reactive oxygen species and enzymes involved in DNA repair [18].

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Conflicts of interest: *none to declare.*

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