Medium-mediated bystander response of X-ray-irradiated normal human lymphocytes *in vitro*

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Abstract. Radiation-induced bystander effects occur in cells that are not directly irradiated but that communicate with irradiated cells via secreted into culture medium soluble factors or gap junction. This effect induces in irradiated and neighboring cells persistent long-term changes that result in delayed death or genomic instability leading to neoplastic transformation. Non-irradiated human normal lymphocytes were incubated in a medium transferred from irradiated another samples of lymphocytes (ICM – irradiation-conditioned medium). Immediately after replacement of the normal or conditioned medium antioxidant vitamins C or E were added. In lymphocytes incubated in a normal medium (control) or conditioned medium, with or without vitamins, the chromosomal damage and apoptosis were estimated. The results show that medium from irradiated cells (ICM) induces in normal lymphocytes micronuclei and condensation of chromatin characteristic of processes of apoptosis. Conditioned medium did not influence cell cycle division. Bystander effect-induced formation of micronuclei was inhibited by antioxidant vitamins C and E, but this had no effect on the induction of apoptosis. These observations suggest that antioxidant vitamins are able to modify radiation-induced bystander effect (by preventing the increase in micronuclei), without inhibiting elimination of damaged cells via apoptosis. This finding could have significant implication for estimating risks of radiation exposure.

Key words: bystander effect • human lymphocytes • antioxidant vitamins • chromosomal damage • apoptosis

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Introduction

Ionizing radiation causes numerous types of DNA damage that occur in exposed cells. Over the past 20 years there has been increasing evidence that cells not directly hit by ionizing track as well as the progent of cells surviving exposure to irradiation can exhibit a wide range of delayed effects called the "bystander effect". It involves genetic changes occurring in cells that were not directly irradiated but responded to signals transmitted from irradiated cells [1, 6, 10, 15]. A number of studies showed that direct cell-cell contact through gap junction communication plays an important role in mediating this phenomenon [1, 17]. There is also evidence for a cell contact-independent bystander effect mediated by soluble factors secreted from irradiated cells into culture medium [4, 16, 12]. It has been shown that medium harvested from irradiated cells induces bystander response in non-irradiated ones and that it is cell type-dependent [8].

The endpoints of the bystander effect include cell killing, induction of chromosome aberrations, micronuclei, gene mutations, altered gene expression and neoplastic transformation [6, 10, 15]. In recent years,

bystander effect has been studied very intensely, however, its mechanism remains largely unknown.

Also, blocking the bystander effect has not been well documented. Modulation of bystander effect may help to understand the mechanisms of this phenomenon.

In the present work we examined the response of human normal lymphocytes to bystander signals emitted from irradiated samples of lymphocytes of the same donor. Since lymphocytes grow in suspension, signals between them should be transmitted by soluble factors released into medium. We examined the response of non-irradiated human normal lymphocytes to signals emmitted from irradiated lymphocytes of the same donors. We tested also the ability of antioxidant vitamins C and E to modulate this response.

Material and methods

Cell cultures and irradiation

The studies were performed on cultures of human peripheral blood lymphocytes obtained from 12 non-smoking volunters ranging from 22 to 26 years of age (5 males, 7 females). All individuals were of similar dietary habits and they were not occupationally exposed to mutagens. Briefly, whole blood cultures were prepared by adding 0.5 ml of blood to 4.5 ml of normal or conditioned RPMI medium supplemented with 15% bovine fetal calf serum and antibiotics. Lymphocytes were stimulated to proliferate by adding phytohaemagglutinin (Lectin, Sigma, 5 μg/ml). For each individual, several duplicate cultures were prepared for the determination of baseline level of micronucleus frequency and for the determination of damage resulting from conditioned medium transferred from irradiated lymphocytes. Normal as well as conditioned medium were supplemented or not, with antioxidant vitamins. All cultures were kept in plastic flasks at 37°C in a humidified 5% CO_2 atmosphere.

X-irradiation was carried out with a Clinac 600 GMV Machine (Varian) using a 2 Gy dose (1 Gy/min).

Preparation of irradiation-conditioned medium (ICM)

Irradiated samples of blood were incubated for 1 h at 37°C and then the medium ICM was transferred to plastic tubes and centrifuged. Next, the supernatant was filtered and used in experiments. The volume of 500 μl of non-irradiated blood was added to 4.5 ml of ICM or normal medium and incubated for required time.

Vitamin C and E treatment

Vitamin C (L-ascorbic acid, Serva) was dissolved in a medium, filtered and added to cultures at a final concentration of 10 μg/ml. Vitamin E (α-tocopherole succinate, Sigma) was dissolved in ethanol and tested at the same concentration as vitamin C. Vitamins were added to cultures of lymphocytes within 10 s after the transfer of the ICM. Experiments included control cultures treated with ethanol alone (10 μl/ml).

Cytokinesis-block micronucleus test

The cytokinesis-block micronucleus test was performed according to a described procedure [3]. Briefly, lymphocytes were stimulated with phytohaemagglutinin (Lectin, Sigma), immediately after adding ICM or normal medium. Cytochalasin B (Sigma, 6 μg/ml) was added 44 h later, to accumulate cells that had divided once. After 72 h incubation, the cultures were harvested and fixed in three changes of methanol/acetic acid (3:1, v/v). The cells were spread onto cold glass slides (two slides for each culture), dried and stained with May-Grunwald and Giemsa dyes. Experiments were repeated on the samples of blood obtained from each donor and the results were summarized. Micronuclei (MN) were scored in 1000 binucleate cells per slide. Data are presented as the frequency of binucleated cells containing one or more MN (MN-CBL). For cell cycle analysis, 400 cells per slide were scored for the presence of one, two, three or more nuclei and the nuclear division index (NDI) was calculated as follows:

$$NDI = [1N + (2 \times 2N) + (3 \times 3N) + (4 \times 4N)]/400$$

where: $1N$ is number of cells with one nucleus; $2N$ – with two nuclei; $3N$ – with three nuclei; $4N$ – with four or more nuclei.

The fraction of cells showing condensation of chromatin characteristic of apoptosis processes was also recorded on the same slides.

Student's t-test was used to determine the statistical significance of differences in the extent of micronuclei frequency between the tested groups in all experiments.

Results

The present study was performed using normal human lymphocytes obtained from 12 healthy donors. The average age of them was 23.3 ± 3.54 (males, $n = 5$) and 23.8 ± 3.84 (females, $n = 7$), respectively. For each donor, the non-irradiated samples of cultured lymphocytes were incubated in normal (control) or conditioned medium transferred from irradiated cultures of lymphocytes and different cellular endpoints were evaluated in control and ICM-treated lymphocytes: micronuclei induction, condensation of chromatin characteristic of apoptosis processes and proliferative capacity of cells measured by nuclear division index.

Data presented in Fig. 1 show that ICM collected from irradiated lymphocytes significantly increased the level of micronucleated binucleate lymphocytes (MN-CBL) and fraction of apoptotic cells. The value of NDI did not markedly differ between lymphocytes incubated in normal or conditioned media.

No increase in micronuclei induction and apoptosis formation was detected when the non-irradiated lymphocytes were cultured with irradiated medium in absence of cells (data not shown).

We examined also the effect of antioxidant vitamins C and E on micronucleus formation and induction of apoptosis in lymphocytes incubated in ICM. Results presented in Fig. 2 show that while both vitamins

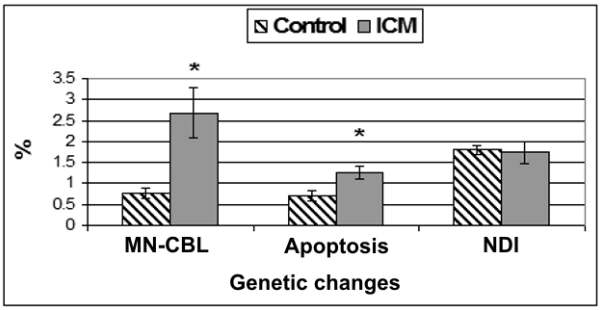

Fig. 1. Induction of micronucleated binucleate lymphocytes (MN-CBL), apoptosis and nuclear division index (NDI) incubated in normal (control) or conditioned medium (ICM). Results are the mean of 12 independent experiments ± SD. * significant at $p < 0.001$ (T-test) compared with lymphocytes incubated in normal (control) medium.

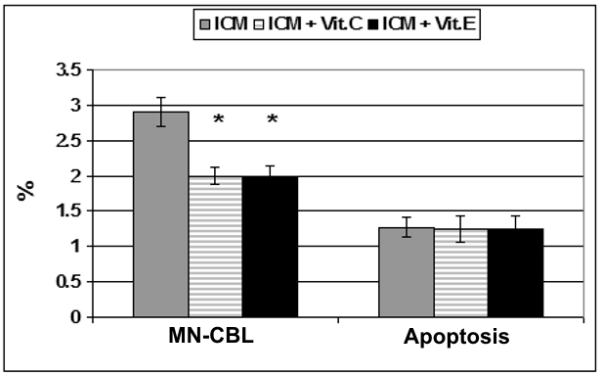

Fig. 2. The influence of vitamin C (10 μg/ml) and vitamin E (10 μg/ml) on the micronuclei formation and apoptosis induction in human lymphocytes incubated in normal (control) or conditioned medium (ICM). Results are the mean of 4 independent experiments ± SD. * significant at $p < 0.001$ (T-test) compared with lymphocytes incubated in ICM without vitamin.

decreased significantly the micronuclei formation, they did not influence the level of apoptosis. Neither vitamin used in indicated concentration nor ethanol in which vitamin E was dissolved, did cause induction of chromosomal damage and apoptosis in normal human lymphocytes (data not shown).

Discussion

The radiation-induced bystander effect occurs in cells that are not directly irradiated but that receive signals from irradiated cells. This effect induces persistent long-term changes that result in delayed death and genomic instability in both irradiated and non-irradiated bystander cells [2, 10]. It was demonstrated in different types of cells [1, 5, 13, 17]. It has been recently demonstrated that irradiation of a single human T lymphocyte with a charged particle leads to a significant induction of chromosomal damage in the bystander populations [7]. In the present study we used the conditioned medium-transfer technique in which the irradiated and non irradiated cells are not cultured together [9]. The results from the present study indicate that human normal lymphocytes exposed *in vitro* to X-ray irradiation are able to release into medium factors leading to induction of bystander response in non-irradiated lymphocytes. It has been shown that irradiated cells release into medium cytokines such as TGFβ and interleukin-8 (IL-8), associated with induction of ROS in cells, several hours after irradiation or transfer of conditioned medium [1, 2, 11, 14]. The results presented in this study demonstrate that addition of antioxidant vitamins C and E decreases the level of micronuclei in lymphocytes growing in ICM, probably via neutralization of secondary biochemical processes linked to the formation of free radicals. In our previous work we have reported that antioxidant vitamins added within one hour after exposure of cultured human lymphocytes to γ-radiation cause radioprotective effect against formation of micronuclei [5]. These results suggest that antioxidant vitamins influence the late phase of radiation action and their protective effect against bystander response observed in present study could be explained by a similar mechanism. The protective action of vitamin C and vitamin E observed in lymphocytes incubated in ICM can be explained by their interaction with radicals or other soluble factors releasing by irradiated cells into medium. These observations may be beneficial for protection of human health after radiation exposure because lymphocytes play an important role in immunological processes. In contrast to the effect described above, apoptosis stimulated by the bystander signals was not inhibited by antioxidant vitamins. This effect suggests involvement of different pathways in inducing bystander response in which scavengers of ROS can participate or not.

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