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ORIGINAL PAPER

Clastogenic effects in human lymphocytes exposed to low and high dose rate X-ray irradiation and vitamin C

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Abstract. In the present work we investigated the ability of vitamin C to modulate clastogenic effects induced in cultured human lymphocytes by X-irradiation delivered at either high (1 Gy/min) or low dose rate (0.24 Gy/min). Biological effects of the irradiation were estimated by cytokinesis-block micronucleus assay including the analysis of the frequency of micronuclei (MN) and apoptotic cells as well as calculation of nuclear division index (NDI). The numbers of micronucleated binucleate lymphocytes (MN-CBL) were 24.85 ± 2.67% and 32.56 ± 3.17% in cultures exposed to X-rays (2 Gy) delivered at low and high dose rates, respectively. Addition of vitamin C (1–20 μg/ml) to the medium of cultures irradiated with the low dose rate reduced the frequency of micronucleated lymphocytes with multiple MN in a concentration-dependent manner. Lymphocytes exposed to the high dose rate radiation showed a U-shape response: low concentration of vitamin C significantly reduced the number of MN, whereas high concentration influenced the radiation-induced total number of micronucleated cells insignificantly, although it increased the number of cells with multiple MN. Addition of vitamin C significantly reduced the fraction of apoptotic cells, irrespective of the X-ray dose rate. These results indicate that radiation dose rate is an important exposure factor, not only in terms of biological cell response to irradiation, but also with respect to the modulating effects of antioxidants.

Key words: vitamin C • X-radiation • dose rate • clastogenic effect

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Introduction

Biological responses to ionizing radiation are complex processes that depend on both the time of exposure and the total absorbed dose. In terms of cell damage at the genetic level, dose rate effects have important implications for both the therapeutic gain and protection of normal tissues during radiotherapy (RT). The risk of harmful genetic effects of irradiation decreases with the reduced dose rate, a phenomenon known as the direct dose rate effect. Generally, acute irradiation is more damaging than the chronic one. High dose rate (> 1 Gy/min) irradiation has been shown to be more effective in inducing genetic damage than low dose rate exposure. It has been demonstrated that by reducing dose rate a decreased MN formation is obtained in human lymphocytes exposed *in vitro* to 0–4 Gy of X-rays at dose rates of 0.2–40 Gy/h [16]. Low dose rate exposure (0.15–1.3 cGy/min) is also less effective in inducing apoptosis in human lymphocytes *in vitro* than high dose rate exposure (70 cGy/min) [3]. It has also been observed that low dose rate irradiation (0–6 Gy/h) is less efficient in inducing MN in human fibroblasts than high dose rate irradiation (78 Gy/h) [7]. These results are also in agreement with other data showing that, compared to high dose rate exposure, low dose

rate irradiation is less effective in inducing mutations in *Drosophila* spermatocytes [10] and teratogenesis in mice [9]. When exposed to low (but not to high) dose rate irradiations some cancer cell lines, however, exhibit an inverse dose rate effect in terms of enhanced sensitivity to cell death induction [13], production of mutations [15], and MN formation [4, 17]. These data indicate that the dose rate effect can depend, as proposed by Hall and Brenner [8], on cell proliferative kinetics and G2-phase block during irradiation.

It has been shown that biological effects of ionizing radiation can be modified by antioxidant vitamins, but there is no evidence of their radioprotective properties with respect to different dose rates of radiation. Several investigators demonstrated that low concentrations of vitamin C decrease endogenous and radiation-induced DNA damage in human lymphocytes, whereas higher concentrations of this vitamin do not offer radioprotection [5, 11, 12, 14].

High dose rate exposures are defined in radiotherapy as those delivered at 1 Gy/min or more, and low dose rate exposures refer to those delivered at less than 1 Gy/min [8].

The present study was conducted to test the ability of vitamin C to modulate the extent of DNA damage in cultured human lymphocytes exposed *in vitro* to X-radiation, delivered at either high (1 Gy/min) or low dose rate (0.24 Gy/min). The DNA damage was estimated using cytokinesis-block micronucleus assay which is useful for evaluating radiation-induced chromosomal damage in peripheral blood lymphocytes [6].

Material and methods

Cell cultures and irradiation

The study was performed using cultures of human peripheral blood lymphocytes obtained from three non-smoking donors. Briefly, whole blood cultures were initiated by adding 0.5 ml of blood to 4.5 ml of RPMI medium (Sigma) supplemented with 15% bovine fetal calf serum and antibiotics. Lymphocytes were stimulated to proliferate by adding phytohemagglutinin (Lectin, Sigma, 5 μg/ml). For each donor, several duplicate cultures were prepared to determine the baseline MN level and chromosome damage following X-irradiation in the presence or absence of vitamin C. X-irradiation was carried out at room temperature with a Clinac 600 GMV Machine (Varian) using a 2 Gy dose delivered at either high (1 Gy/min) or low (0.24 Gy/min) dose rate. All experiments were repeated three times.

Vitamin C treatment

Vitamin C (L-ascorbic acid, Serva) was dissolved in culture medium, filtered and added to the cell cultures at a final concentration from 1 to 20 μg/ml, 30 min before irradiation.

Cytokinesis-block micronucleus test

The cytokinesis-block micronucleus test was performed according to a described procedure [6]. Briefly, lymphocytes were stimulated with phytohemagglutinin immediately after irradiation. 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 with the samples of blood obtained from each donor and the results pooled. Micronuclei (MN) were scored in 500 binucleate cells (BN) per slide. Data are presented as the frequency of binucleated cells containing one or more micronuclei, as well as micronuclei distribution. For the 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:

$$(1) \qquad \%\mathrm{NDI} = [1N + (2 \times 2N) + (3 \times 3N) + (4 \times 4N)] / 400$$

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

The number of cells showing condensation of chromatin that is characteristic for apoptosis was also scored on the same slides.

Student's t-test was used to determine the statistical significance of differences in the extent of chromosomal damage between the sets irradiated in the presence or in the absence of vitamin C.

Results

Cultured human lymphocytes were exposed to 2 Gy of X-ray delivered either at a high dose rate (HDR, 1 Gy/min) or at a low dose rate (LDR, 0.24 Gy/min), in the presence or absence of vitamin C. The radiation-induced biological effects were estimated as the frequency

Table 1. Effect of vitamin C treatment on NDI values in lymphocytes irradiated with X-rays at different dose rates (the data represent mean ± SD)

Vitamin C treatment	NDI (%)	
	Dose rate 0.24 (Gy/min)	Dose rate 1 (Gy/min)
Not treated	1.72 ± 0.06	
Irradiated only	1.57 ± 0.06	1.60 ± 0.04
1 μg/ml	1.58 ± 0.04	1.66 ± 0.07
5 μg/ml	1.54 ± 0.05	1.64 ± 0.06
10 μg/ml	1.57 ± 0.07	1.66 ± 0.05
20 μg/ml	1.53 ± 0.06	1.62 ± 0.05

Table 2. Distribution of MN[a] in BN[b] expose in the presence of witamin C (1 or 20 μg/ml) to X-rays at high or low dose rates

Treatment			MN distribution (MN/1000 cells)				
Dose (Gy)	Dose rate (Gy/min)	Concentration of witamin C	1 MN	2 MN	3 MN	4 MN	Total
0	0	0	22.80	2.78	0.60	0.00	30.16
0	0	20	26.00	2.00	0.00	0.00	30.00
2	0.24	0	187.00	39.00	5.50	0.50	283.50
2	0.24	1	188.00	29.00	4.50	0.00	295.50
2	0.24	20	153.00	23.75	2.00	0.00	206.50
2	1	0	201.00	56.60	28.33	6.00	423.19
2	1	1	152.33	25.67	2.33	0.00	210.66
2	1	20	212.67	45.00	33.00	9.00	437.67

[a] MN – micronuclei. [b] BN – binucleated cells.

of MN and condensation of chromatin characteristic for apoptosis as well as the change in proliferative capacity of cells determined by the NDI. Table 1 presents the effect of vitamin C on the proliferative capacity of cells, calculated as NDI of human lymphocytes exposed to X-radiation at two different dose rates. The NDI values decreased from 1.72 in non-irradiated (control) cells to 1.60 or 1.57 in cells exposed to 2 Gy, at LDR or HDR, respectively. Vitamin C did not markedly influence the value of NDI in lymphocytes, independent of radiation dose rate. We observed a trend for lower values of NDI following radiation exposure delivered at LDR, as compared with HDR, however, the change was not statistically significant.

The modulating effect of vitamin C on the number of MN induced in lymphocytes by X-radiation at different dose rates is presented in Fig. 1. Vitamin C at each concentration tested did not influence the level either of MN or NDI (data not shown). In lymphocytes irradiated in the absence of vitamin C we observed a greater number of micronucleated cells following HDR radiation exposure, as compared to LDR.

The level of MN in lymphocytes exposed to HDR was efficiently reduced by vitamin C only at a low concentration (below 5 μg/ml); at higher concentrations vitamin C was not effective as a radioprotector. On the contrary, in cultures exposed to LDR of X-radiation, vitamin C was ineffective at the concentration of 1 μg/ml, but above 5 μg/ml and up to 20 μg/ml it showed a concentration--dependent protective effect.

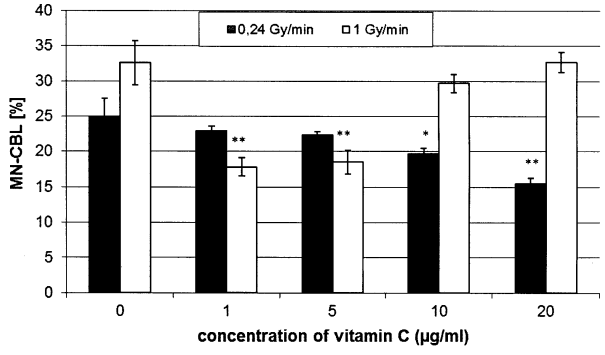

Fig. 1. The effect of vitamin C on the number of micronucleated lymphocytes exposed *in vitro* to 2 Gy of X-rays at different dose rates. Results are means ± SD for three experiments. * $p < 0.05$; ** $p < 0.01$ refers to differences between cells irradiated only and cells preincubated with vitamin C (Student's t-test).

We also analyzed the modifying effect of vitamin C on the distribution of MN following exposure to different radiation dose rate (Table 2). Low dose rate radiation elevated predominantly the number of cells with one micronucleus whereas high dose rate radiation caused an increase in the number of the BN cells with more than one MN. Vitamin C had an impact on the MN distribution.

When lymphocytes were exposed to low dose radiation, vitamin C diminished the number of micronucleated cells. The effect was pronounced more strongly at 20 μg/ml than at 1 μg/ml. We observed a decreased formation of cells with more than one micronucleus.

In cells exposed to HDR radiation vitamin C at 1 μg/ml had a strong protective effect against formation of cells with more than one micronucleus. Vitamin C at high concentration (20 μg/ml) increased the number of BN cells with 3 and 4 MN, although the total number of micronucleated cells was changed insignificantly by vitamin C.

The effects of vitamin C on the number of cells showing condensation of chromatin characteristic for processes of apoptosis in lymphocytes irradiated with different dose rates are presented in Fig. 2. The results show that X-radiation induced a comparable effect in terms of apoptosis induction when applied at 0.24 Gy/min or 1 Gy/min dose rates. Vitamin C, independent of its concentration, caused a decrease in the number of lymphocytes showing chromatin condensation, both in cells exposed to low and to high dose rates of X-radiation.

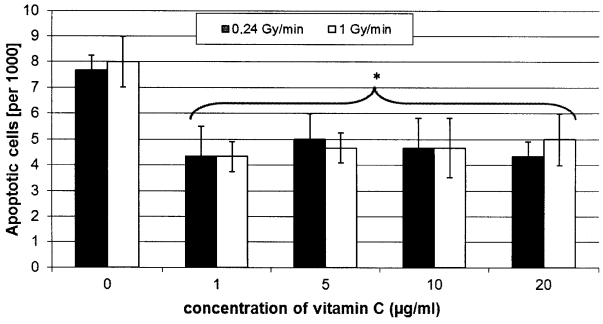

Fig. 2. The effect of vitamin C on the number of lymphocytes showing apoptotic morphological changes induced by X-rays at different dose rates. Results are means ± SD for three experiments. * $p < 0.05$ refers to differences between cells irradiated only and cells preincubated with vitamin C (Student's t-test).

Discussion

In the present study we compared the modulating effect of vitamin C on the micronucleus formation and on induction of apoptosis in human lymphocytes exposed *in vitro* to X-irradiation delivered at two different dose rates.

The results of the micronucleus assay indicate that the HDR (1 Gy/min) of X-radiation induced *in vitro* more micronuclei in cultured human lymphocytes than the LDR (0.24 Gy/min). This observation is in agreement with the published data indicating that the level of chromosomal damage is lower with the decreased dose rate in irradiated human lymphocytes [3, 16] and fibroblasts [7], but it does not agree with other reports indicating that LDR is more effective in cell killing [13] and micronuclei formation [17]. Recently, a decreasing dose rate effect in TK6 human lymphoblastoid cells exposed to X-rays at variable dose rates was shown [4], but the authors examined the 0.025 to 0.0042 Gy/min range which is lower than the dose rates used by us. Differences between the discussed results can be due to different cell types used in the experiments. The level of DNA damage in irradiated cells is influenced by several factors such as oxygenation status, chromatin conformation, cell signaling and repair capacity [1].

It has generally been accepted that reduction in the dose rate leads to an accumulation of cells in the G1 and G2 phases of the cell cycle, that is mediated by the transducer protein kinase ATM, which prevents the cells from replicating damaged DNA and provides time for DNA repair or apoptosis induction in the damaged cells [8]. The result of our study confirms the above-described data and indicates that the LDR causes a moderate inhibition of the proliferative capacity of lymphocytes expressed as NDI (1.57 ± 0.06), when compared to the HDR (1.60 ± 0.04).

The micronucleus assay results indicate that the reduction of the dose rate of X-radiation substantially changes the modulating effect of vitamin C. This observation is partially consistent with our previous report showing that vitamin C at a low concentration acted as a radioprotector, whereas at a high concentration it potentiated the clastogenic action of ionizing radiation, the latter effect was observed when cells were exposed only to a HDR (1 Gy/min) radiation [11, 12]. In the present work we also observed a protective effect of vitamin C at 1 μg/ml but a radiosensitizing effect at 20 μg/ml. The data presented in Table 2 show that vitamin C at 20 μg/ml insignificantly changes the radiation-induced total number of micronuclei in binucleated cells. However, from the distribution of micronuclei it follows that an increase in the number of cells with many MN takes place. A new finding from the present study is that the decrease in the dose rate from 1 Gy/min to 0.24 Gy/min causes a change in the modulating effect of vitamin C, which shows radioprotective properties in a concentration-dependent manner. The variable effect of vitamin C depending on the dose rate of radiation can result from a balance between the radiation-induced free radicals and the prooxidant/antioxidant properties of ascorbic acid. It seems possible that under LDR conditions of irradiation the oxygen radicals can be sufficiently scavenged by vitamin C and this effect increases with the vitamin concentration. Under HDR irradiation conditions a high concentration of vitamin C can enhance the production of hydroxyl radicals from hydrogen peroxide via Fenton reaction, according to the published data [2]. Higher numbers of the reactive oxygen species induced at higher a dose rate probably cause an additive damaging effect exerted by vitamin C.

With respect to apoptosis induction no significant differences were observed in relation to the different dose rates of X-radiation. Vitamin C, independent of its concentration, caused a significant decrease in the number of lymphocytes showing apoptotic morphology, similar to the one seen in cultures exposed to low and high dose rates. The lack of dose rate effect on apoptosis induction observed in the present study is in agreement with the cited evidence that the dose rate effect of radiation for apoptosis induction can be observed only at a very LDR (0.15 cGy/min) [3].

In our previous paper we demonstrated that the level of apoptosis induced by radiation does not necessarily correlate with the induction of micronuclei. In this study we found that vitamin C did not affect the level of radiation-induced apoptosis in human lymphocytes; instead, it significantly reduced the number of DNA breaks measured by the comet assay [12].

These results suggest that vitamin C influenced the extent of the radiation-induced DNA breakage and the repair process but not the apoptosis-dependent pathways.

The present study confirms the earlier reports indicating that the dose rate is an important parameter of ionizing radiation when considering the biological responses of irradiated cells. It also shows that the protective effect of an antioxidant vitamin depends not only on its concentration but also on the irradiation conditions. This is especially important for the innovative radiation oncology solutions based on various types of the conventional and intensity modulated radiotherapy (IMRT).

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