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Role of arachidonic acid metabolism in thymocyte apoptosis after irradiation

Yuri N. Korystov*, Oksana R. Dobrovinskaya, Vera V. Shaposhnikova, Lazar Kh. Eidus

Laboratory of Cell Radiobiology, Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino 142292, Russian Federation

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Abstract The present study demonstrates that DNA fragmentation, nuclear pycnosis and trypan blue staining of irradiated thymocytes is prevented by inhibition of the lipoxygenase pathway of arachidonic acid metabolism and is not affected by cyclooxygenase inhibition. Exposed to irradiation [^{3}H]arachidonic acid-labeled thymocytes release radioactive products to the external medium. The process is blocked by the lipoxygenase inhibitor, nordihydroguaiaretic acid. Thus, it can be concluded that irradiation activates arachidonic acid metabolism and that lipoxygenase metabolites play an important role in thymocyte apoptosis.

Key words: Irradiation; Thymocyte apoptosis; Arachidonic acid metabolism

1. Introduction

The biochemical regulation of programmed cell death (apoptosis) has recently attracted a great attention because of its role in many normal processes and pathological conditions [1,2]. Apoptosis can be initiated by various inducers such as corticoids, quinone compounds, by triggering of cytokine receptors, deprivation of growth factors and ionizing radiation [3]. The apoptotic death of irradiated thymocytes is dependent on their interactions occurring via as yet unknown mediators [4,5]. The AA metabolites are likely candidates for this role. These compounds are of great importance in the regulation of different immune reactions where the interactions between specialized cells take place [6–8]. A variety of cell types involved is known to have extracellular specific receptors to AA metabolites [9,10]. PLA_2, a catalyzer of the hydrolysis of choline and ethanolamine glycerophospholipid, is the key enzyme responsible for AA release at different signal reactions in mammalian cells [11–13]. The released AA is consequently oxidized either by cyclooxygenase with formation of cyclic products like prostaglandins, or by lipoxygenase giving rise to linear chain products such as lipoxines and leukotrienes [8,13]. Previously we have shown that BPB, a PLA_2 inhibitor [11], prevents nuclear pycnosis [5] and DNA fragmentation [14] in irradiated thymocytes. Nuclear pycnosis was the only criterion of the effects of lipoxygenase and cyclooxygenase inhibitors [5]. Nuclear pycnosis (or chromatin condensation) and internucleosomal DNA fragmentation are known to have different metabolic pathways [15,16]. Therefore different criteria of apoptosis are needed to determine the role of metabolic pathways in the whole process.

The purpose of this work is to study the effects of cyclooxygenase and lipoxygenase inhibitors on DNA fragmentation, chromatin condensation and membrane damage in irradiated thymocytes as well as the kinetics of AA metabolites release from thymocytes after irradiation.

2. Materials and methods

Thymocytes were isolated from male Wistar rats (130–150 g) by a standard procedure [4]. Cells were suspended in Hanks' balanced salt solution with 20 mM HEPES at 37°C, washed once, and resuspended in Hanks' solution to obtain a final concentration of 1.5×10^7 cells/ml. Then the cells were irradiated with ^{60}Co γ-rays at a dose rate of 3.2 Gy/min at room temperature. The postirradiation incubation was carried out in 96-well flat-bottomed plates at 200 μl/well at 37°C in Hanks' solution. We abandoned postirradiation incubation of thymocytes in nutrient media (RPMI 1640) as this medium greatly decreased the cell death tested with trypan blue [15].

Cell death was evaluated by the decrease in cell number (cell lysis) and by staining of cells in a 0.04% solution of the trypan blue dye (Serva) after 24 h incubation [4]. Pycnotic cells were identified with acetic acid–ethanol (1:3) fixation and Giemsa staining after 6 h incubation.

DNA fragmentation was determined 6 h after postirradiation incubation according to [17]. Cells (approximately 1.5×10^7 cells) were scraped from culture plates, isolated by centrifugation for 10 min at $700\times g$, resuspended in 1 ml of a lysis buffer containing 5 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0, and allowed to lyse for 15 min on ice. Samples were then centrifuged at $13\,000\times g$ for 25 min to separate high molecular weight chromatin (pellet) from cleavage products (supernatant). Pellets were resuspended in 1 ml of buffer solution containing 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. Pellets and supernatant were assayed for DNA content using the diphenylamine reagent [18]. DNA staining with diphenylamine was performed at 30°C for 17–20 h. DNA content was evaluated from absorption at a wavelength of 600 nm. The percentage of DNA fragmentation was determined as the ratio of optical density in the supernatant to the sum of optical densities in the supernatant and the pellet.

The damage of irradiated thymocytes was modified by addition of indomethacin and NDGA, purchased from Serva (Heidelberg, Germany). Stock solutions of indomethacin and NDGA in DMSO were used. These compounds were used at conventional concentrations [19,20]. They were added into the cell suspensions prior to irradiation and allowed to remain there throughout the incubation period: 6 h for determination of nuclear pycnosis and DNA fragmentation and 24 h for determination of the cell death.

The thymocytes (1.5×10^7 cells/ml) were labelled in 3 ml RPMI 1640 medium supplemented with 1% bovine serum and 1 μCi/ml [^{3}H]arachidonic acid (150 Ci/mM; 6.7 nM) for 1 h at 37°C as described previously [21,22]. After the incubation, the supernatant was removed, and the cells were washed three times with RPMI 1640 supplemented with 0.5% albumin and once with Hanks' solution. The incorporation of radioactivity into the cells approximated 2.5×10^4 cpm per 10^6 cells. Labelled cells were exposed to irradiation, and cell suspensions in Hanks' solution ($5–10\times10^6$ cells/ml) were incubated over different periods of time in glass tubes at 37°C and then

*Corresponding author.

Abbreviations: A23187, calcium ionophore; AA, arachidonic acid; BPB, 4-bromophenacylbromide; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; NDGA, nordihydroguaiaretic acid; PLA_2, phospholipase A_2

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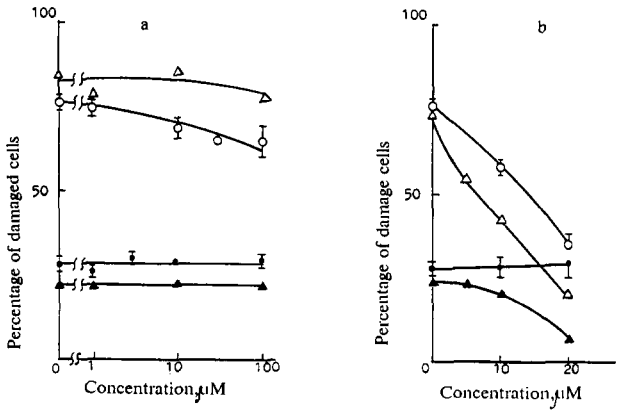

Fig. 1. Effects of indomethacin (a) and NDGA (b) on cell death (△, ▲) and nuclear pycnosis (○, ●) of 6 Gy irradiated (○, △) and control (●, ▲) thymocytes; for the cell death $n = 2$ and for nuclear pycnosis $n = 4$.

were centrifuged at $4000 \times g$ for 5 min. Radioactivity released into the medium in the course of cell incubation was evaluated by counting of the supernatant aliquots (150 μl). Just prior to incubation the radioactivity release was 325 ± 35 cpm per 10^6 cells which was taken as reference point and this value was subtracted from all the subsequent values. Points in the graphs are means ± standard deviation ($n > 3$). The number of assays (n) for each experiment is indicated in the figure legends.

3. Results and discussion

As shown in Figs. 1b and 2, NDGA, an inhibitor of the lipoxygenase pathway [23], decreased nuclear pycnosis, cell death and DNA fragmentation in a dose dependent manner. NDGA decreased cell death and DNA fragmentation in control also at concentrations above 20 μM. At the same time, indomethacin, which is known to cause specific blockage of cyclooxygenase at a concentration of 1 μM [19], had no effect on all apoptosis criteria up to a concentration of 100 μM (Fig. 1a, Fig. 2). To exclude antioxidant activity of NDGA [24] in apoptosis we checked the effects of other antioxidants. Ionole (10^{-4} M), α-tocopherol (6×10^{-4} M), and ascorbic acid (10^{-4} M) did not affect DNA fragmentation (data not shown). This control confirmed that NDGA affects apoptosis via lipoxygenase inhibition. Taken together these data and the apoptosis prevention with the PLA_2 inhibitor [5,14] suggest involvement of the lipoxygenase branch of AA metabolism in radiation-induced and in vitro incubation-induced apoptosis of thymocytes. Involvement of the lipoxygenase pathway in tumor cell apoptosis mediated by tumor necrosis factor was recently shown [25].

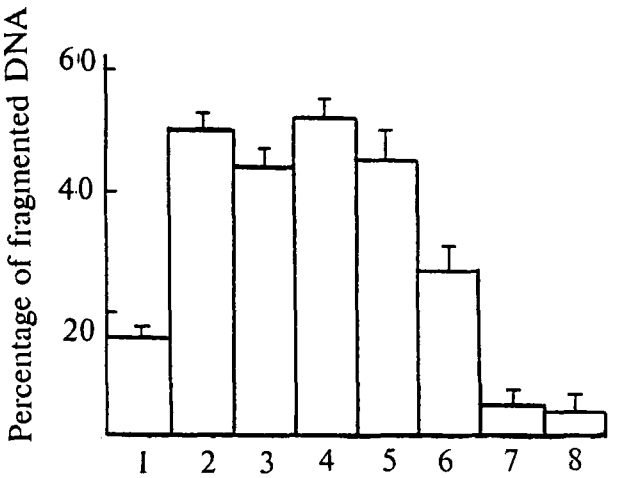

Fig. 2. Effects of indomethacin and NDGA on DNA fragmentation. 1, control; 2, 6 Gy; 3, 6 Gy+1 μM indomethacin; 4, 6 Gy+10 μM indomethacin; 5, 6 Gy+100 μM indomethacin; 6, 6 Gy+20 μM NDGA; 7, 40 μM NDGA; 8, 6 Gy+40 μM NDGA. n = 3–6. Indomethacin had no effect on DNA fragmentation in control; 20 μM NDGA decreased DNA fragmentation insignificantly, up to 13 ± 4%.

The radiation activation of AA metabolism was confirmed using a complementary approach, by measurements of radioactivity release from irradiated thymocytes preincubated with ^{3}H-labeled AA. Calcium ionophore A23187 is known to be an activator of PLA_2 and AA release from membrane [21]. Hence, treatment with 0.1 μM A23187 was used as a control to support that ^{3}H-labeled AA had been incorporated into membrane. The radioactivity release from thymocytes treated with A23187 was about 3000 cpm per 10^6 cells 60 min after stimulation, which was approximately 10–12% of the total radioactivity incorporation, being in agreement with [21]. The kinetics of radioactivity release from 6 Gy irradiated thymocytes isolated from different rats varied greatly. The different types of responses are shown in Fig. 3. In seven experiments out of 24 the radioactivity release was close to that in the control. In other cases the release of labeled products was observed, and reached its maximum at 30–60 min after irradiation.

The data variability could be caused partly by changes in some experimental conditions from day to day. Therefore four rats were examined in one day. Nuclear pycnosis and death of thymocytes after irradiation were found to be identical for all the rats tested, but irradiation stimulation of radioactivity release was observed in thymocytes from three rats only with a maximum at 30 min (two rats) and 60 min (one rat), which was 3–4 times more than the control level. The absence

Table 1
Effect of NDGA (20 μM) on ^{3}H-radioactivity release by irradiated thymocytes[a]

Time	^{3}H-radioactivity release (cpm/10^6 cells)						
(min)	control[c]	Exp. 1		Exp. 2		Exp. 3	
		6 Gy	6 Gy+NDGA	6 Gy	6 Gy+NDGA	6 Gy	6 Gy+NDGA
30	69 ± 15	169	113	8217	0[b]	237	82
60	108 ± 20	168	95	257	33	162	0[b]

[a]The data of three experiments with significant ^{3}H-radioactivity release after irradiation are presented.
[b]The values are the same as those obtained prior to incubation (325 ± 35 cpm/10^6 cells).
[c]The values given are the averages of 24 experiments ± SD.

of an increase in labeled products in the medium after irradiation of thymocytes could be explained by the enhanced capacity of the thymocytes to reabsorb AA in this case. To check it we used fatty acid free albumin which is known to suppress cell reabsorption of AA and its metabolites [26]. The addition of albumin to the thymocyte suspension strongly increased the radioactivity release, being more pronounced for irradiated cells (Fig. 4). The release kinetics was changed to a monotonic one in the presence of albumin. These data show that radiation stimulates AA metabolism in thymocytes from all rats but in some cases the effect is not revealed in the absence of albumin. The cell reabsorption of AA and its metabolites is caused by AA reacylation [26] and binding of AA metabolites to the receptors at the cell surface [9,10]. Variations in these processes in the thymocytes from different rats remain an open question, but such variation is in the usual run of things when the radioactivity release from irradiated lymphoid cells preincubated with labeled AA was studied in incubation medium [27].

The addition of NDGA to the cells with pronounced release of labeled products prevented this process (Table 1). This implies that the bulk of releasing products could be lipoxygenase metabolites. For example, in platelets 10 s after stimulation only 20–25% of ^3H label liberated was still the free acid, the rest having been converted to products of cyclooxygenase and lipoxygenase [26], and lipoxygenase products predominantly [28]. In addition the decreased release of lipoxygenase metabolites in the presence of inhibitor can suppress [^3H]AA release also because PLA_2 activity depends on protein kinase C [29] and leukotrienes activate the latter [30]. The kinetics of radioactivity release is also consistent with the behavior of lipoxygenase metabolites found with the activation of leukocytes and lymphocytes by other stimuli. The maximal release was usually observed 5–10 min after cell activation, whereas most of the products remained bound to the cells or reabsorbed after release by the receptors at the surface of the cell membrane [10,31,32].

In conclusion, the work presented here shows that irradiation of thymocytes leads to activation of AA metabolism, and that lipoxygenase metabolites play an important role in radiation-induced thymocyte apoptosis.

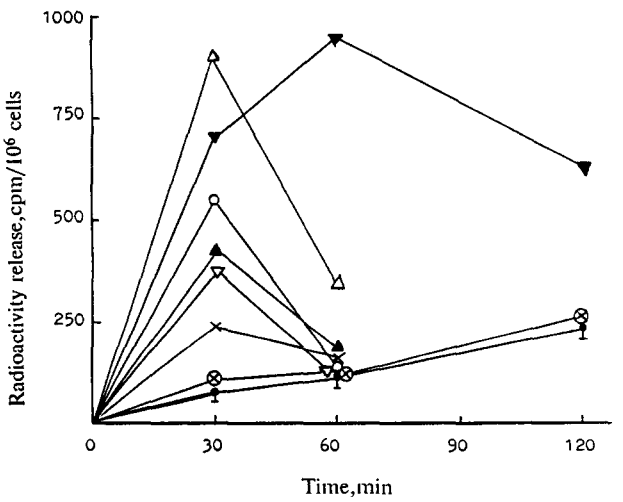

Fig. 3. Time course of radioactivity release by 6 Gy irradiated thymocytes. The zero time point is taken in the beginning of incubation at t = 37°C. Values of radioactivity release by unirradiated thymocytes (●) are plotted as mean ± SD (n = 24). The radioactivity release by irradiated thymocytes is presented as data of individual experiments; the values given are the averages of duplicates.

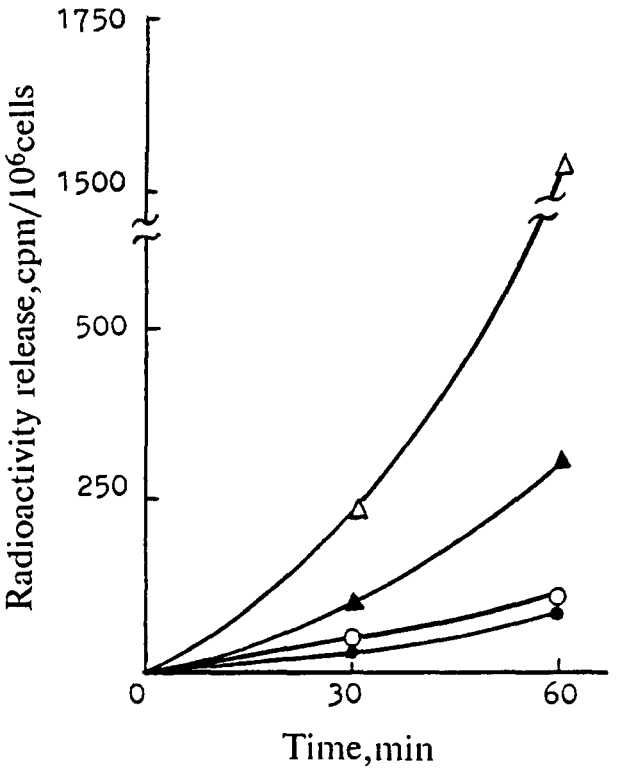

Fig. 4. Time course of radioactivity release by control (●, ▲) and 6 Gy irradiated (○, △) thymocytes in Hanks' solution (●, ○) and that supplemented with 40 mg/ml fatty acid free albumin (▲, △).

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