The effect of melittin on proliferation and death of thymocytes

V.V. Shaposhnikova, M.V. Egorova, A.A. Kudryavtsev, M.Kh. Levitman, Yu.N. Korystov*

Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Moscow Region, Pushchino 142292, Russia

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Abstract The effect of melittin, an activator of phospholipase A_2, on proliferation and death of rat thymocytes in a broad concentration range was studied. Cell proliferation was estimated by the accumulation of colchicin metaphases, necrotic death was determined from lysis and staining of cells with trypan blue, and apoptosis was assessed from the type of DNA fragmentation, the amount of fragmented DNA, and the percentage of cells with subdiploid DNA. It was shown that low melittin concentrations (below 5 μg/ml) stimulate thymocyte proliferation. At high melittin concentrations, thymocytes die by the primary necrosis type. Throughout the concentration range studied, melittin does not produce apoptosis in thymocytes. Conversely, high melittin concentrations even inhibit thymocyte apoptosis in the control and after irradiation. An inhibitor of RNA synthesis actinomycin D does not affect thymocyte death in the presence of melittin. It is concluded that the activation of phospholipase A_2 can induce necrosis but not apoptosis and thus is not a necessary step in the signaling cascade that initiates apoptosis in thymocytes.

Key words: Apoptosis; Melittin; Necrosis; Phospholipase A_2; Proliferation; Thymocyte

1. Introduction

Membrane phospholipids are the source of various intra- and intercellular messengers, which control and mediate proliferation, death, and other cellular responses [1,2]. One class of messengers possessing a broad spectrum of biological action is leukotriens, the products of oxidation by lipoxygenase of arachidonic acid, which results from the reaction of PLA_2 with phospholipids [3]. Previously, we have shown that apoptosis of thymocytes induced by radiation proceeds with the participation of PLA_2, lipoxygenase, and protein kinase C because the inhibitors of these enzymes suppressed apoptosis [4–6]. A possible scheme of transduction of the signal that triggers the apoptosis in thymocyte after irradiation was proposed [7]. However, the inhibition analysis only indicates the involvement of an enzyme in the process and does not provide an answer to the question of whether the activation of the enzyme is required for the realization of the effect. As for protein kinase C, it was reported that it can be activated after irradiation [8] and that phorbol ester, a specific activator of protein kinase C, is able to induce and enhance apoptosis in thymocytes [6]. A direct examination of PLA_2 activation after irradiation of thymocytes from the release of radioactive products from cells labeled with [^{3}H] arachidonic acid gave ambiguous result [5]. First, the time course of the radioactivity release showed a maximum, indicating either the reverse incorporation of the label or its binding. Second, the release of labeled products was almost entirely suppressed by the inhibitor of lipoxygenase, i.e. the label was released predominantly in the form of lipoxygenase products. Thus, the increased release of the label from thymocytes after irradiation might be due to the activation of lipoxygenase only, and the question of whether PLA_2 is activated during apoptosis remained open. To answer this question, we used another approach in this study: we examined the effect of melittin, a component of bee venom that specifically activates PLA_2 [9], on thymocytes. It is known that high concentrations of melittin induce cell death by the necrosis type [9], and whether melittin can induce apoptosis was not studied earlier.

2. Materials and methods

Thymocytes were isolated from male Wistar rats (140–160 g) by a standard procedure [4]. Cells (10^7/ml) were incubated in RPMJ-1640 medium supplemented with 10% bovine serum, 10 mM HEPES and 10 μg/ml of gentamycin at 37°C for 24 h. Cell death (necrosis) was estimated by a decrease in cell number (cell lysis) and by staining of cells in a solution of 0.04% trypan blue dye (Serva):

$$\text{percentage of dead cells} = \frac{(N_0 - N_1) + N_2}{N_0} \times 100\%$$

where N_0 is the initial concentration of unstained cells, N_1 is the final concentration of unstained cells, and N_2 is the final concentration of stained cells. This method of estimating cell death is applicable when there is no increment in cell number ($N_1 + N_2 < N_0$). If cell concentration increases during incubation due to division, the term 'death' is inadequate to characterize the state of population. Therefore, under certain conditions (Fig. 1), the criterion of cell survival was used:

$$B = \frac{N_1}{N_0} \times 100\%$$

where N_1 is the final concentration of unstained cells, which can be higher and lower than N_0, depending on the extent of proliferation and death of cells in the population.

The extent of DNA fragmentation was determined by the method of Perotti [10]. Cells (1.5×10^7) were precipitated for 5 min by centrifugation at $700 \times g$ and lyzed for 15 min on ice with 0.5% Triton X-100 containing 5 mM Triton-HCl and 20 mM EDTA (pH 8). Samples were then centrifuged for 25 min at $13000 \times g$ to separate the high-molecular weight DNA (pellet) from the degradation products (supernatant). Pellets were suspended in a solution containing 10 mM Tris-HCl and 1 mM EDTA (pH 8).

DNA was stained with diphenylamine at 30°C for 20 h [11]. The DNA content was determined spectrophotometrically by absorbance at $\lambda = 600$ nm. The percentage of fragmented DNA was defined as the ratio of optical density in the supernatant to the sum of optical densities in the supernatant and pellet.

Electrophoresis for determining fragmented DNA was carried out in agarose gel by the method of Asakawa [12]. Washed cells (10^6) were lysed for 3 h at 50°C in a solution (20 μl) containing 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% SDS, and 2 μg/ml of pronase (Sigma). After the addition of 10 μl of 0.5 μg/ml of RNAse A (Sigma), cells were incubated for an additional hour. Samples treated with pronase and RNAse were stained with 0.25% bromophenol blue (Serva) for 10 min at 70°C during incubation in a solution containing 10 mM EDTA

*Corresponding author. Fax: (7) (95) 924-04-93.

Abbreviations: PLA_2, phospholipase A_2; EDTA, ethylene diamine tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid

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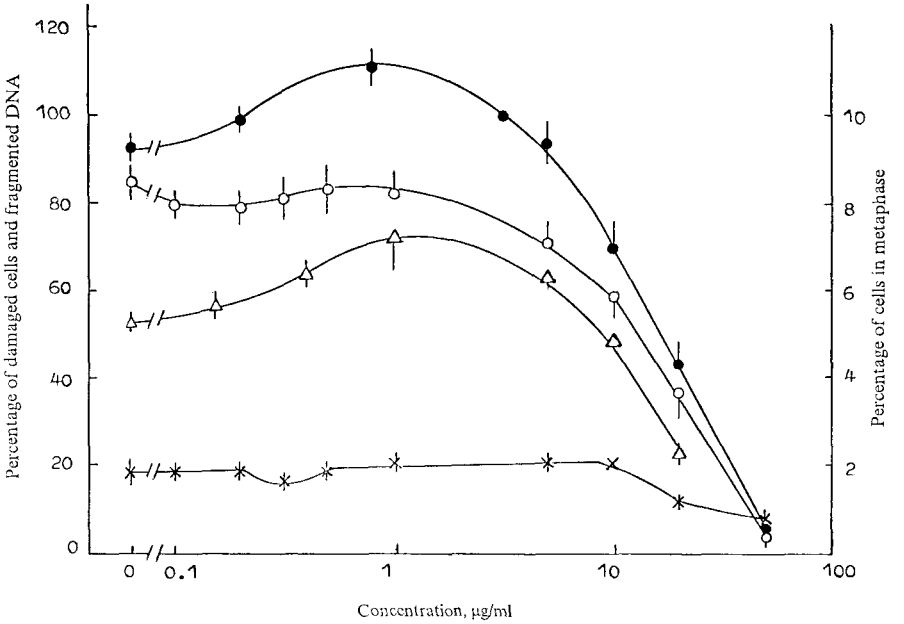

Fig. 1. Effect of melittin on DNA fragmentation (×), metaphase index (△) and cell survival after 6 (●) and 24 (○) h incubation of thymocytes.

(pH 8.0) and 40% sucrose. DNA was separated in 2% agarose gel for 4 h at $U=5$ V/cm. After staining with ethidium bromide, gels were photographed in UV light.

Cell proliferation was defined as the percentage of metaphases present in the incubation medium after a 6-h incubation of cells with colchicin (3×10^{-5} M, Fluka) [13]. The metaphase frequency was calculated under microscope at a 600-fold magnification by examining 1–2 thousands of cells in each preparation fixed in a mixture of methanol and acetic acid (3:1) and stained by the method of Giemse.

The percentage of cells with the subdiploid set of DNA was determined on a flow cytometer (laboratory set-up). Cells fixed with cold 70% ethanol were stained, transferred into phosphate buffer (pH 7.2), and stained with Hoechst 33258 (Serva) for 5 min (2 μg/ml). In each series, 20–50 thousands of cells were assayed. The relative amount of cells with the DNA content corresponding to different stages of the cycle was determined from experimental histograms.

Melittin (Sigma) and actinomycin D (Serva) were added to the thymocyte suspension prior to incubation. Irradiation of cells was carried out with ^{60}Co γ-rays, at a dose rate of 2.1 Gy/min at room temperature.

The point in the graphs are the means ± standard deviation. The number of assays (n) for each experiment per one series is given in the figure legends.

3. Results and discussion

Fig. 1 shows the effect of melittin on survival, DNA fragmentation, and metaphase index of thymocytes as a function of dose. At concentrations below 5 μg/ml, melittin stimulates thymocyte proliferation, which is evidenced by an increase in the portion of colchicine metaphases and in survival as compared to control. The increase in survival after a 6-h incubation is more pronounced than after a 24-h incubation. The difference may be due to both an increase in cell death with increasing duration of melittin treatment and the fact that thymocytes undergo presumably only one division under these conditions, with the duration of the mitotic cycle being close to 6 h [14]. As melittin concentration increases, the metaphase index and survival gradually decrease; at a concentration of about 50 μg/ml, almost all cells die even after six hours of incubation. At all concentrations studied, an increase in DNA fragmentation, which is a criterion of apoptosis [15], does not take place; on the contrary, at concentrations causing cell death, melittin inhibits DNA fragmentation in the control. The results obtained indicate that a low activation of PLA_2 stimulates thymocyte proliferation, whereas a strong activation induces death by the primary necrosis type since apoptosis, which precedes the secondary necrosis, does not take place over a wide range of melittin concentrations (0.1–50 μg/ml) and hence PLA_2 activity. It is known that the intensity of treatment affects the response of heterogeneous cell populations. In particular, low doses of ionizing radiation stimulate thymocyte proliferation, whereas high-dose radiation initiates apoptosis [16]. The change in the total response of cell population depending on treatment intensity is likely to be due to the fact that different cell types have different intensity thresholds of the signal that triggers the reaction specific for this very cell type. Evidently, this threshold is lower for proliferat-

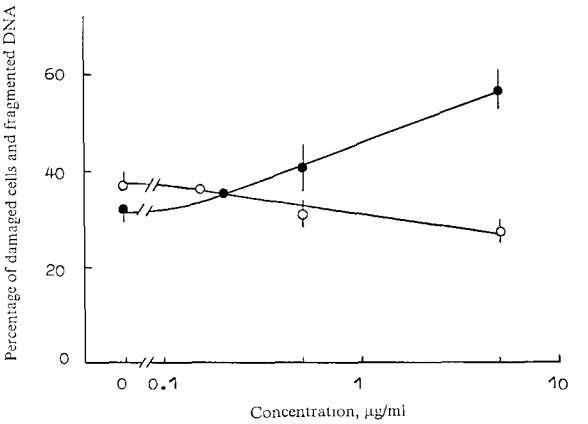

Fig. 2. Effect of melittin on DNA fragmentation after 6-h incubation (○) and cell death after 24-h incubation (●) of thymocytes irradiated with a dose of 0.5 Gy.

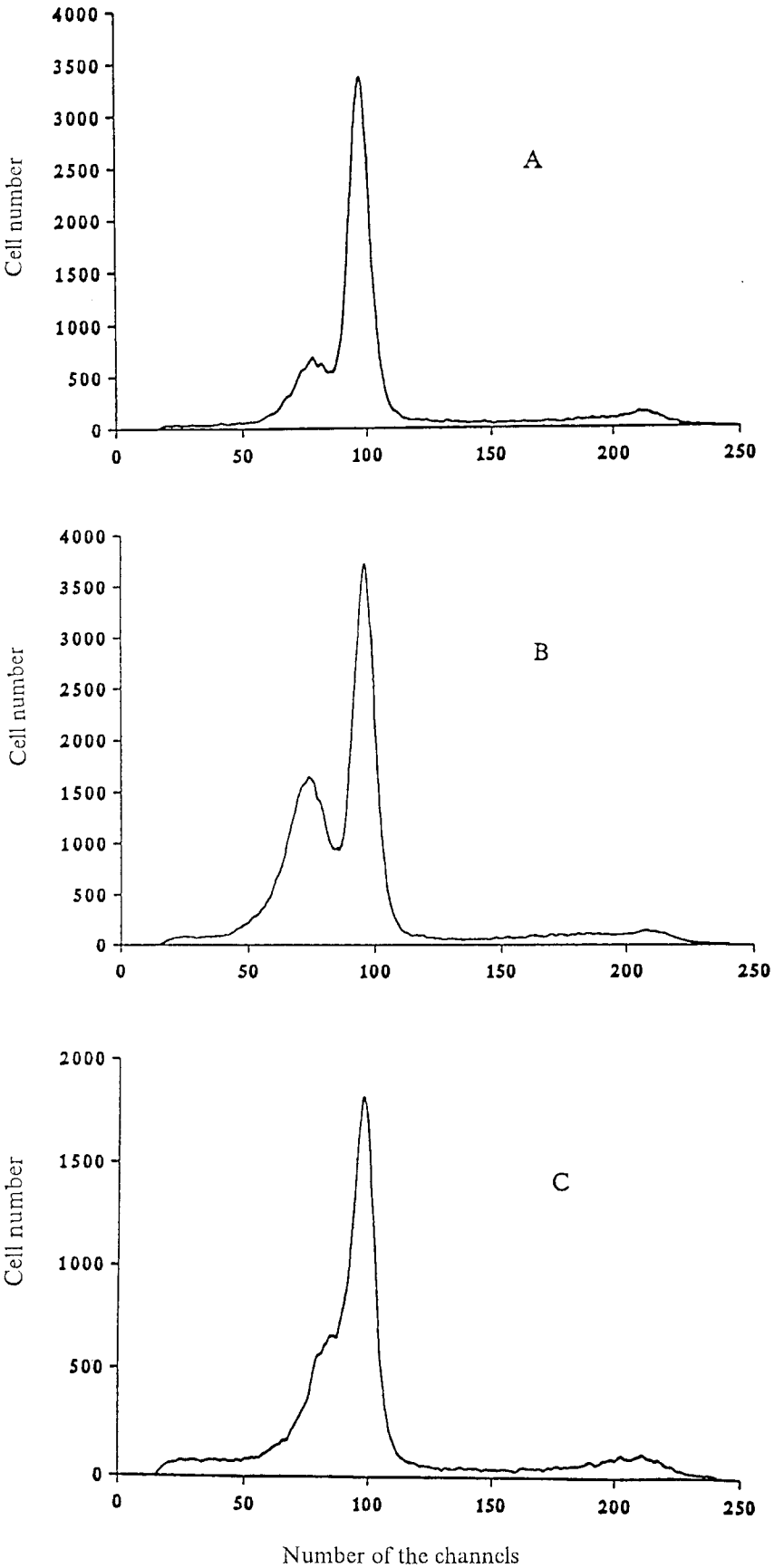

Fig. 3. Distribution of thymocytes with respect to DNA content in control (A), after irradiation with a dose of 0.5 Gy (B), and after treatment of irradiated cells (0.5 Gy) with melittin (5 μg/ml) (C). The content of DNA in G_1 phase is in channels $N \sim 100$ and content of DNA in G_2, M phases is in channels $N \sim 200$.

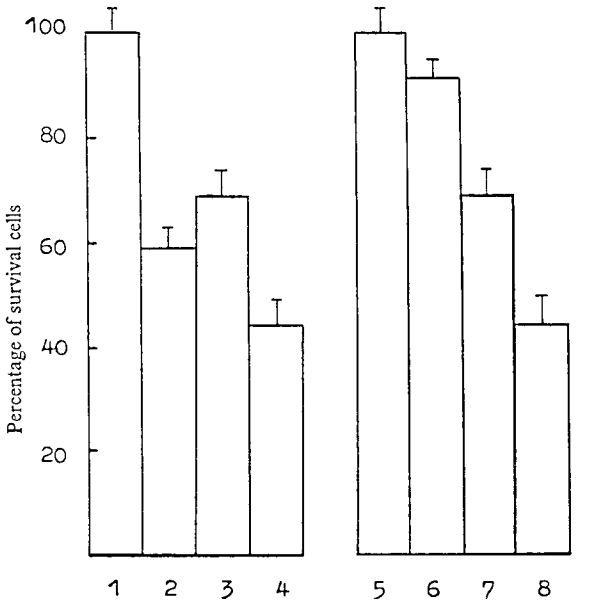

Fig. 4. Effect of actinomycin D on the survival of irradiated (6 Gy) and melittin-treated thymocytes after 24-h incubation. On the ordinate is the cell survival relative to its control. 1, control; the survival in the control, which is equal to 85±4% relative to the initial concentration, is assumed to be 100% for variants 2–4; 2, irradiation with a dose of 6 Gy; 3, in the presence of 10 μg/ml of melittin; 4, 20 μg/ml of melittin; 5, 5 μg/ml of actinomycin D; the survival in the presence of actinomycin D, which is equal to 65±4% relative to the initial concentration is assumed to be 100% for variants 5–8; 6, irradiation with a 6 Gy dose +5 μg/ml of actinomycin D; 7, 10 μg/ml of melittin +5 μg/ml of actinomycin D; 8, 20 μg/ml of melittin +5 μg/ml of actinomycin D.

ing thymocytes than for differentiating cells most of which are destined to die.

In the next set of experiments, we studied the effect of melittin on the apoptosis induced in thymocytes by another treatment, the ionizing radiation. Fig. 2 shows the dose dependencies of the effect of melittin on death and DNA fragmentation in irradiated thymocytes. It is seen that as melittin concentration increases, the percentage of cell death increases and that of DNA fragmentation decreases. The inhibition of apoptosis by melittin is confirmed by the method of DNA electrophoresis (data not shown). Fig. 3 shows the distribution of cells with respect to DNA content, as determined by flow cytometry. After irradiation, the portion of apoptotic cells with subdiploid DNA increases from 22% to 40% (Fig. 3A and B); melittin reduces the percentage of irradiation-induced apoptosis of cells to 33% (Fig. 3C). The inhibition of thymocyte apoptosis in the control and after irradiation is likely to be due to the fact that the normal metabolism, which governs the signal transduction and transcription of genes initiating apoptosis, is disturbed at necrotic damage to plasma membrane (exit of metabolites from the cell, changes in ion distribution, suppression of energy production, etc.). Therefore, as the level of the necrotic death increases, the apoptosis is suppressed (see, for example, Fig. 2). Previously, we have shown that incubation of irradiated thymocytes under conditions enhancing the necrotic form of death leads to the inhibition of DNA fragmentation [17]. The antagonism of necrosis and apoptosis was also shown on other cell types exposed to different influences [18,19].

As it is known, apoptosis differs from necrosis primarily in that for its realization the activation and transcription of genes are necessary [20]. Therefore, based on the dependence of thymocyte apoptosis on RNA or on protein synthesis [21], it is possible to classify the type of death by its essential feature and not formally. Fig. 4 shows the effect of an inhibitor of RNA synthesis, actinomycin D, on the survival of irradiated and melittin-treated thymocytes. The results are presented relative to the control since actinomycin D by itself at a concentration of 5 μg/ml reduced survival by 20% during the 24-h incubation. Both irradiation and melittin induce cell death; actinomycin D does not affect the melittin-induced death but almost completely inhibits the death induced by irradiation. DNA fragmentation in irradiated (6 Gy) thymocytes is also reduced by the action of actinomycin D, from 54±4 to 16±6, i.e. to the value below the control. These results indicate that thymocyte death produced by melittin, as opposed to radiation-induced death, is of the primary necrosis type that is not associated with gene activation and RNA synthesis and is likely to be due to the direct effect of activated PLA_2 on the membrane.

Thus, the activator of PLA_2 at low concentrations stimulates proliferation, and at large concentrations, it induces thymocyte necrosis which is accompanied by inhibition of apoptosis in control or after irradiation. Considering that the inhibitor of PLA_2 blocks apostosis of thymocytes [4,6], and the activator of PLA_2 does not initiate it, it can be concluded that for apoptosis in rat thymocytes, the basic activity of PLA_2 is necessary and sufficient, and that activation of PLA_2 is not an obligatory step in the signalling cascade that induces apoptosis in thymocytes.

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