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Dependence of thymocyte apoptosis on protein kinase C and phospholipase A_2

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Abstract

The effect of inhibitors and activators of protein kinase C and phospholipase A_2 on radiation-induced apoptosis of rat and mouse thymocytes has been studied. It is shown that the apoptosis is prevented by the protein kinase C inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperasine dihydrochloride and is potentiated by protein kinase C activator phorbol 12-myristate 13-acetate, calcium ionophore A23187 and concanavalin A. The protein kinase C activators initiate apoptosis in mouse but not in rat thymocytes. The inhibitor of phospholipase A_2 prevents apoptosis induced by all the factors. The results obtained indicate that both protein kinase C and phospholipase A_2 are involved in the thymocyte apoptosis.

Key words: Irradiation; Thymocyte apoptosis; Protein kinase C; Phospholipase A_2

1. Introduction

Death of thymocytes characterized by internucleosome DNA fragmentation, may occur due to either negative selection of autoreactive clones of T-cells [1] or treatment by various specific effectors [2]. In any cases, apoptosis was shown to require activation of some genes and synthesis of macromolecules [1,2]. The cells possess several signaling systems that are responsible for the gene activation [3]. There is evidence pointing to the involvement of PTK-PKC-dependent signaling pathway in radiation-induced activation of genes in lymphoid cells leading to DNA fragmentation. Thus, it was demonstrated that radiation stimulates the activity of PTK and PKC [4,5] while the inhibitors of PTK and PKC prevent radiation-induced apoptosis [6,7]. It was also shown that PKC activation during apoptosis is PTK-dependent [7].

The signal transduction from PTK to PKC may occur in different ways: (i) through activation of PLC-γ and elevating $[Ca^{2+}]_i$ [8]; (ii) through activation of PLA_2 [9], the resulting in formation of arachidonic acid and the lipoxygenase metabolites that may activate PKC directly [10] and indirectly through inhibition of GAP leading to an increase in the Ras-GTP level [11,12] responsible for PKC activation [13]. Previously we were able to demonstrate that during the thymocyte apoptosis caused by ionizing radiation, the $[Ca^{2+}]_i$ was not increased within 1 h, i.e. up to appearance of morphological changes characteristic of apoptosis [14], while the inhibitors of PLA_2 and lipoxygenase completely prevented apoptosis [15]. These data imply that during radiation-induced apoptotic death of thymocytes the signal transduction from PTK aod PKC may occur by the second mechanism. The necessity of PKC and PLA_2 activity for apoptosis was shown for radiation only, and this was based on the experiments envolving inhibitors [6,7]. Another approach, the modification of apoptosis by activators of this enzymes, has not been used until present. The role PKC and PLA_2 in apoptosis induced by other agents has not been studied either. The present work is undertaken to study the effects of inhibitors and activators of PKC and PLA_2 on apoptosis of rat and mouse thymocytes after radiation and dexametazon treatment.

2. Materials and methods

Thymocytes were isolated from male Wistar rats (140–160 g) and male $F_1 \times C_{57}$ BL/6 mice (22–24 g) by a standard procedure [15]. The cells were suspended in RPMI 1640 medium supplemented with 10% bovine serum, 10 mM HEPES and then irradiated with ^{60}Co γ-rays at a dose rate of 3 Gy/min at room temperature. Activators and inhibitors of PKC and PLA_2 were added to cells suspension immediately before irradiation. For this, the following compounds were used: PMA (Sigma), arachidonic acid (Sigma), concanavalin A (Serva), A23187 (Sigma), BPB (Sigma), H7 (Serva). Apoptosis was estimated as DNA fragmentation and nuclear pycnosis. Pycnotic cells were identified by Giemsa staining after fixation with acetic acid–ethanol (1:3) on the 6th hour of post-irradiation incubation at 37°C. DNA fragmentation was estimated by the method reported elsewhere [16]. The cells (1.5×10^7) harvested by centrifugation for 5 min at $700 \times g$ were lysed in 0.5% Triton X-100 containing 5 mM Tris-HCl (pH 8), 20 mM EDTA for 15 min on ice. The samples were then centrifuged at 13000 g to separate

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Abbreviations: A23187, calcium ionophore; BPB, 4-bromophenacylbromide; $[Ca^{2+}]_i$, cytosolic concentration of Ca^{2+}; GAP, GTPase activating protein; H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperasine dihydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperasineethane-sulfonic acid; IP_3, inositol triphosphate; PIP_2, phosphoinositol 4,5-biphosphate; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PTK, protein-tyrosine kinase; PKC, protein kinase C; PLA_2, phospholipase A_2.

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high- molecular-weight chromatin (pellet) from cleavage products (supernatant). Pellets were resuspended in 1 ml of a buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. Pellets and supernatants were assayed for DNA content using the diphenylamine reagent [17]. DNA staining with diphenylamine was performed at 30°C during 17–20 h. DNA content was evaluated from absorption at a wavelength of 600 nm. The percentage of fragmented DNA was determined as the ratio of the optical density in the supernatant to the sum of optical densities in the supernatant and the pellet.

3. Results and discussion

As it was previously shown [6,18], H-7, a protein kinase C inhibitor, prevents DNA fragmentation in thymocytes after apoptosis-induced radiation. A similar effect of H-7 was revealed by the criterion of nuclear pycnotization during radiation- and dexametazon-induced apoptic death of rat thymocytes (Fig. 1). If the radiation-induced apoptotic death requires an activation of PKC, the PKC activators must potentiate the radiation effect. As seen from Fig. 2, activators of PKC, such as PMA [19] and arachidonic acid [10] do stimulate DNA fragmentation in thymocytes after radiation. Response of thymocytes to the PKC activators by the DNA fragmentation criterion appears to be species-dependent. Fig. 3 illustrates the data on the effect of PMA on the radiation-induced mouse thymocytes apoptosis. It is seen that in irradiated mouse thymocytes, like in rat thymocytes, PMA potentiates DNA fragmentation. However, the mouse thymocytes appeared more susceptible to the PKC activators. Without radiation, 10 nM PMA induces DNA fragmentation in mouse but not rat thymocytes. Another way of PKC activation involves the increase of $[Ca^{2+}]_i$. It is known that exposure to concanavalin A or calcium ionophore A23187 elevates the Ca^{2+} concentration in thymocytes [20]. Treatment of mouse thymocytes with concanavalin A (5 μg/ml) does not stimulate DNA fragmentation but potentiate the PMA effect (Fig. 3). Concanavalin A (20 μg/ml) induces DNA fragmentation in mouse (Fig. 4). In rat thymocytes, this concentration of concanavalin A causes a less pronounced effect (Fig. 2). Slight increase in DNA fragmentation can be also induced by A23187 (Fig. 4). An elevated susceptibility of mouse thymocytes to PKC activators is likely determined by a greater basal activity of PKC.

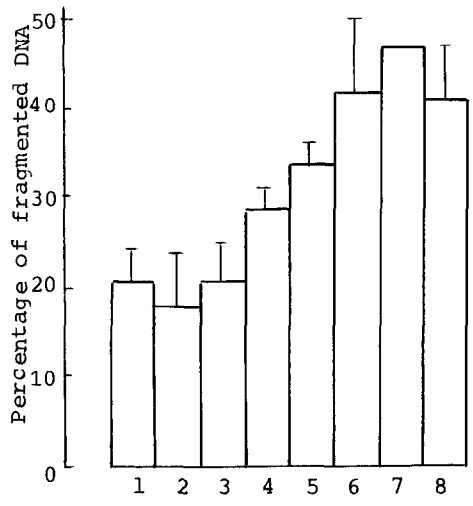

Fig. 2. Effect of PKC activators on DNA fragmentation of control and irradiated rat thymocytes after 6 h incubation. 1 = control; 2 = 10 nM PMA; 3 = 50 μM arachidonic acid; 4 = concanavalin A (20 μg/ml); 5 = 2 Gy; 6 = 2 Gy + 10 nM PMA; 7 = 2 Gy + 2 μM arachidonic acid; 8 = 2 Gy + 50 μM arachidonic acid.

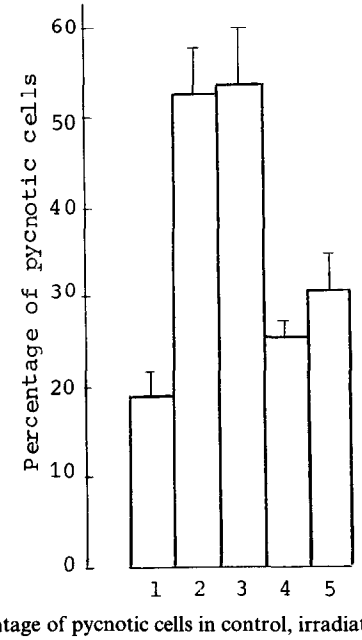

Fig. 1. Percentage of pycnotic cells in control, irradiated and dexametazon-treated thymocytes after 6 h incubation. 1 = control; 2 = 6 Gy; 3 = 100 nM dexametazon; 4 = 6 Gy + 50 μM H7; 5 = 100 nM dexametazon + 50 μM H7.

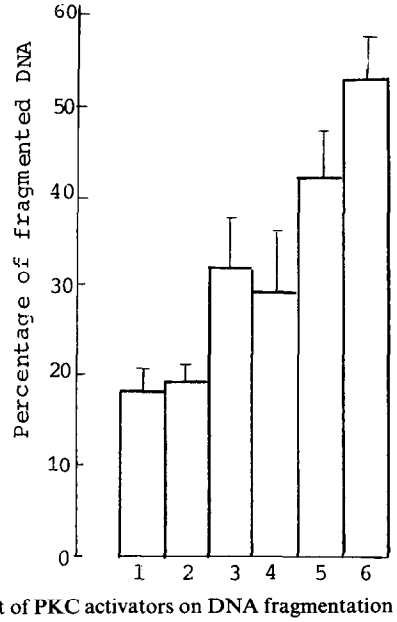

Fig. 3. Effect of PKC activators on DNA fragmentation of control and irradiated mouse thymocytes after 6 h incubation. 1 = control; 2 = concanavalin A (5 μg/ml); 3 = 0.5 Gy; 4 = 10 nM PMA; 5 = concanavalin A (5 μg/ml) + 10 nM PMA; 6 = 0.5 Gy + 10 nM PMA.

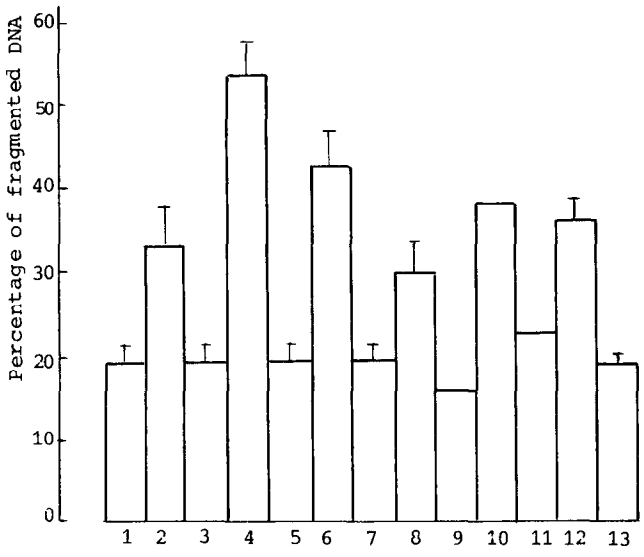

Fig. 4. Effect of BPB (3 μg/ml) on DNA fragmentation in mouse thymocytes induced by various agents. 1 = control; 2 = 0.5 Gy; 3 = 0.5 Gy + BPB; 4 = 0.5 Gy + 10 nM PMA; 5 = 0.5 Gy + 10 nM PMA + BPB; 6 = concanavalin A (5 μg/ml) + 10 nM PMA; 7 = concanavalin A (5 μg/ml) + 10 nM PMA + BPB; 8 = 100 nM A23187; 9 = 100 nM A23187 + BPB; 10 = concanavalin A (20 μg/ml); 11 = concanavalin A (20 μg/ml) + BPB; 12 = 100 nM dexametazon; 13 = 100 nM dexametazon + BPB. 1–7,10,11 = mouse thymocytes; 8,9,12,13 = rat thymocytes.

The data summarized in section 1 indicate that during radiation-induced apoptotic thymocyte death, the PKC activation requires the involvement of PLA_2 but not calcium-dependent PLC pathway. It appeared that the activity of PLA_2 is required for both potentiation of radiation-induced apoptosis by PKC activators and its initiation by the PKC activators themselves, including the calcium ionophore A23187. Fig. 4 presents the data indicating a complete inhibition of thymocyte DNA fragmentation by the PLA_2 inhibitor BPB under different conditions. Since PMA (diacylglycerol analog) and A23187 can activate PKC with no PLA_2 involved, dependence of apoptosis induced by these agents on the PLA_2 activity suggests that DNA fragmentation requires an extensive and/or continuous activation of PKC, which is mediated by PLA_2. In other words, PLA_2 seems to be a necessary signal amplifier. Probably, the signal amplifications occur by means of positive feedbacks between PKC and PLA_2. It was shown [21] that phosphorylation of the phospholipase inhibitory protein lipomodulin at serine residues leads to its inactivation and eventually to PLA_2 activation. In turn, the products of PLA_2 and lypoxigenase arachidonic acid metabolism induce activation of PKC [10]. Thus, the results obtained indicate that thymocyte apoptosis induced by various agents requires the involvement of both PKC and PLA_2. Interaction of these enzymes will probably result in the formation of a positive feedback capable of a prolong maintenance of a high activity of PKC and PLA_2 necessary for apoptosis.

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