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On the role of intracellular concentration of Ca^{2+} and H^+ in thymocyte death after irradiation

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The role of intracellular Ca^{2+} and H^+ concentrations in radiation-induced interphase death of rat thymocytes has been studied. In response to concanavalin A treatment in the Ca^{2+}-containing medium, or to the $CaCl_2$ treatment in the Ca^{2+}-free medium, the $[Ca^{2+}]_i$ rise in irradiated cells was as in the non-treated cells. No changes in the level of $[Ca^{2+}]_i$ and pH_i were found within 1 h after irradiation of thymocytes with a dose of 6 Gy. 15 μM 5-(*N*-ethyl-*N*-isopropyl)-amiloride, an inhibitor of Na^+/H^+ exchange, did not affect the DNA fragmentation. The fragmentation was prevented by 2–4 μM (1-[bis(4-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)]-2-[(2,4-dichlorophenyl)-methoxy]-ethyl)-1-*H*-imidazolium chloride, an inhibitor of calmodulin. The above data indicate that triggering of interphase death in irradiated thymocytes is not mediated by changes in either $[Ca^{2+}]_i$ or pH_i. Such changes seem to be involved in intermediate steps of the interphase death process.

Irradiation; Thymocyte death; Intracellular concentration of Ca^{2+} and H^+ ions

1. INTRODUCTION

The change in intracellular concentration of calcium ($[Ca^{2+}]_i$) and pH_i is an essential event in the activation of a variety of physiological processes such as secretion of hormones and neurotransmitters, muscle contraction, cell proliferation, etc. [1–3]. Cell death, the apoptotic death of thymocytes, in particular, induced by glycocorticoids or ionizing radiation, is also a Ca^{2+}-dependent process [4–6]. Interphase death of irradiated thymocytes depends on both Ca^{2+} and pH in the medium [7]. However, the role of intracellular concentration of these ions in thymocyte death remains obscure. Two possible ways are discussed: the death triggering, or the death realization (the development of damage) is Ca^{2+}- and pH-dependent.

Radiation-induced death of thymocytes shows a lag time of about 1 h [8,9]. Within this period cell death was not revealed, but different biochemical processes leading to the cell death are being activated. If the change in $[Ca^{2+}]_i$ or pH_i triggered radiation-induced thymocyte death, one should expect any changes in the concentration of these ions during the lag period. The scarce literature data on the role of $[Ca^{2+}]_i$ in initiating thymocyte death are contradictory. McConcey et al. [10] showed an increase in $[Ca^{2+}]_i$ 1 h after pretreatment of thymocytes with methylprednisolone. However, similar experiments of Kotelewskaya et al. [4] showed no $[Ca^{2+}]_i$ rise, in contradiction to the supposed mediatory role of $[Ca^{2+}]_i$ rise in the triggering of the death of methylprednisolone-treated thymocytes. A twofold $[Ca^{2+}]_i$ rise 30 min after irradiation of thymocytes was reported in [11] but was not observed for irradiated lymphocytes of peripheral human blood [12].

The present study is an attempt to elucidate whether triggering of death in irradiated thymocytes is mediated by Ca^{2+} and H^+ or whether the ions are involved in its realization.

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Abbreviations $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; pH_i, cytosolic pH; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BCECF, 2′,7′-biscarboxyethyl-5(6)-carboxyfluorescein; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; Con A, concanavalin A; R 24571, (1-[bis(4-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)]-2-[(2,4-dichlorophenyl)-methoxy]-ethyl)-1-*H*-imidazolium chloride, calmidazolium; Quin-2, 2-[2-bis(carboxy-methylamino-5-methylphenoxy)-methyl]-6-methoxy-8-bis(carboxy-methyl)aminoquinoline, TPA, 12-*O*-tetradecenoylphorbol-13-acetate

2. MATERIALS AND METHODS

Thymocytes were isolated from male Wistar rats (140–160 g) by a standard procedure. The cells were suspended in Hank's balanced salt solution supplemented with 20 mM HEPES and then irradiated with ^{60}Co γ-rays at a dose rate of 3 Gy/min at room temperature. The post-irradiation incubation was carried out at 37°C in 96-well flat-bottomed plates at a concentration of $1–2 \cdot 10^7$ cells/ml.

Cell death was estimated as DNA fragmentation by the method reported elsewhere [13]. The cells ($1.5 \cdot 10^7$) harvested by centrifugation for 5 min at $700 \times g$ were lysed in 0.5% Triton X-100 containing

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5 mM Tris-HCl (pH 8), 20 mM EDTA for 15 min on ice. The samples were then centrifuged at 13,000 × g to separate 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 [14]. DNA staining with diphenylamine was performed at 30°C during 17–20 h. DNA content was evaluated from absorption at 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.

To measure $[Ca^{2+}]_i$, the cells ($1 \cdot 10^8$ ml^{-1}) were incubated at 37°C for 40 min in Hank's solution containing 10 μM Quin-2 acetoxymethyl ester [15]. For pH_i measurements the cells were incubated at 37°C for 30 min with 4 μM BCECF acetoxymethyl ester [16]. The thymocytes were then washed twice, placed in a medium free of the dyes and irradiated with γ-rays at room temperature. The fluorescence was measured in the laboratory-made spectrofluorimeter [17] at 37°C either immediately after irradiation (on the average after 8 min at room temperature) or after 30 and 60 min incubation of cells at 37°C. Control cells were incubated in a similar fashion. The excitation and emission wavelengths were 337 and 495 nm for Quin-2 and 500 and 530 nm for BCECF, respectively. The $[Ca^{2+}]_i$ and pH_i values were calculated as in [18,19].

R24571 (Serva) and EIPA (Sigma), inhibitors of calmodulin and Na^+/H^+ exchange, respectively, were used to modify the death of irradiated thymocytes.

3. RESULTS AND DISCUSSION

Fig. 1 shows absence of $[Ca^{2+}]_i$ rise in irradiated cells during 1-h incubation at 37°C. By the end of this period the fluorescence intensity tends to increase both in control and in irradiated cells, which is probably related to the Quin-2 release during cell incubation. In some assays 6 Gy-irradiated thymocytes were incubated at 37°C in a fluorimeter chamber. Within 15 min after irradiation, the level of $[Ca^{2+}]_i$ was not significantly changed, being on average 116 ± 8 nM (121 ± 18 nM in control cells). The addition of 1 mM $CaCl_2$ to the Ca^{2+}-free medium brings about the response of $[Ca^{2+}]_i$ in irradiated cells the same as in control ones (Fig. 2B). In normal Ca^{2+} medium, the response of $[Ca^{2+}]_i$ to Con A (15 μg/ml) was the same in irradiated and unirradiated cells (Fig. 2A). By and large these data imply that during 1 h after irradiation, the systems of intracellular Ca^{2+} homeostasis provide a permanent $[Ca^{2+}]_i$ level.

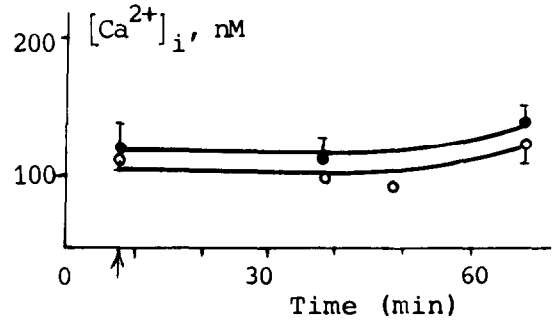

Fig. 1 $[Ca^{2+}]_i$ level in control (●) and 6 Gy-irradiated (○) thymocytes during post-irradiation incubation; the arrow indicates the onset of incubation at 37°C.

It is known that the death of irradiated thymocytes depends on the temperature and is effectively realized only above 37°C. Lowering the temperature to 20°C strongly reduces the $[Ca^{2+}]_i$ response to Con A, TPA and monensin [20]. Therefore, a transient $[Ca^{2+}]_i$ rise is hardly possible during irradiation of cells at room temperature (20°C). Nevertheless we performed the experiments involving irradiation of thymocytes on ice (0°C). The rise in $[Ca^{2+}]_i$ has not been really observed.

In contrast to these data, a slight increase in $[Ca^{2+}]_i$ was shown in rat thymocytes 30 min after irradiation in [11]. This discrepancy may be related to peculiarities of the cells used. Indeed a low level of $[Ca^{2+}]_i$ in control cells (50 nM instead of 115 ± 5 nM [17,18]) and a more rapid death of control and irradiated cells as compared to literature values [21] suggest that the physiological state of the cells used in this study differs from the norm.

Thus our experimental data show that triggering the interphase death of irradiated thymocytes is not mediated by a $[Ca^{2+}]_i$ rise.

As the radiation-induced death of thymocytes is a Ca^{2+}-dependent process it can be suggested that Ca^{2+} act as a co-factor of enzymes such as Ca-Mg-dependent endonuclease which brings about chromatin fragmentation, or phospholipase A_2 producing lysophosphatides that destroy the cell membrane [22,23]. It is known also

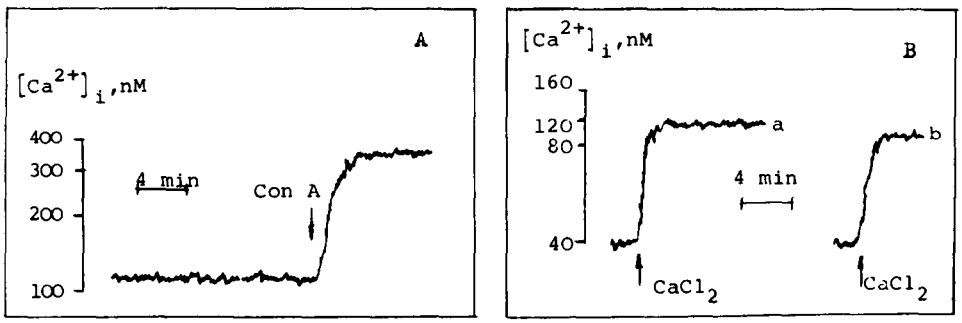

Fig. 2. (A) Change in $[Ca^{2+}]_i$ in irradiated rat thymocytes induced by the addition of Con A (15 μg/ml). (B) Change in $[Ca^{2+}]_i$ in irradiated (a) and control (b) thymocytes induced by addition of 1 mM $CaCl_2$ to the Ca^{2+}-free incubation medium. The thymocytes were loaded with Quin-2, washed and irradiated at 20°C. After a 30-min incubation at 37°C the cells were placed in thermostated chamber of spectrofluorimeter (37°C) and changes in the fluorescence intensity were monitored.

Table I

The effect of calmodulin inhibitor R24571 and Na^+/H^+ exchange inhibitor EIPA on DNA fragmentation of thymocytes (control and irradiated (6 Gy)) after a 6-h incubation at 37°C

Inhibitor	–	R24571		EIPA
Concentration (μM)	–	2	4	15
% of fragmented DNA in control	23 ± 2	21 ± 1	26 ± 6	20 ± 4
% of fragmented DNA after irradiation (6 Gy)	52 ± 4	39 ± 3	34 ± 2	48 ± 4

Table II

pH_i of control and irradiated (6 Gy) thymocytes after a 30-min incubation at 37°C (results of 2 experiments)

	Time after irradiation (min)	
	0	30
Control	7.22 (7.23)	7.18 (7.21)
6 Gy	7.20 (7.19)	7.19 (7.20)

that the majority of enzymic reactions in the cell are activated not by Ca^{2+} ions themselves but by their complex with calmodulin, an intracellular receptor for these ions [24].

Thus, under calmodulin inhibition, death could also be suppressed in the presence of Ca^{2+}. It is known that antagonists of calmodulin can block DNA fragmentation in thymocytes under cold shock [13] and methylprednisolone treatment [10]. Table I shows that 2–4 μM R24571, an inhibitor of calmodulin, also prevents DNA fragmentation in irradiated thymocytes after a 6-h incubation at 37°C.

The role of pH_i in triggering thymocyte death was assessed by blocking Na^+/H^+ exchange. The cell response to the stimulus should be reduced if the increase in pH_i is an essential event in this reaction. Table I summarizes the data indicating that EIPA, an inhibitor of the Na^+/H^+ exchange, has no effect on the DNA fragmentation in irradiated thymocytes. Hence, interphase death of thymocytes cannot be mediated by pH_i either. This conclusion was supported by direct pH_i measurements in irradiated thymocytes. It can be seen from Table II that pH_i does not change within 30 min after exposure of thymocytes to 6 Gy. The known dependence of thymocyte death on the medium pH probably accounts for the activation of the enzymes involved in cell damage development (e.g. phospholipase A_2), since the change in the medium pH is accompanied by a change in pH_i, though to a lesser extent [25].

Thus, the presented data show no change in the $[Ca^{2+}]_i$ and pH_i levels during the lag period after irradiation of thymocytes. These ions probably mediate death realization rather than death triggering.

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