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Investigation of Calcium Accumulation in Mitochondria in Cells Undergoing Apoptosis

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Abstract—One of the earliest features of apoptosis is the induction of the mitochondrial permeability transition (MPT) due to opening of a pore in the mitochondrial membrane. We estimated the Ca^{2+} capacity of mitochondria (a threshold level of Ca^{2+} that induces the release of this cation from mitochondria) during apoptosis. Incubation of thymocytes at 37°C for 4 h equally decreased the mitochondrial Ca^{2+} capacity both in the presence and the absence of dexamethasone, an inducer of apoptosis. At the same time, dexamethasone significantly stimulated internucleosomal DNA fragmentation, which is one of the manifestations of apoptosis. Cyclosporin A prevented the time-dependent decrease in the Ca^{2+} capacity of mitochondria but did not affect internucleosomal DNA fragmentation. Therefore, induction of apoptosis assessed by internucleosomal DNA fragmentation is not mediated by the mitochondrial permeability transition.

Key words: apoptosis, mitochondria, calcium, permeability transition

Apoptosis, or the programmed cell death is a gene-regulated form of cell self-destruction [1, 2]. Certain morphological and biochemical features of apoptosis separate it from another form of cell death—necrosis. Among these features are chromatin condensation, cell shrinkage, DNA internucleosomal fragmentation, etc. [3-5]. One of the characteristic manifestations of apoptosis is a decrease in the potential on the inner mitochondrial membrane. This decrease has been putatively explained as being due to the opening of a pore and induction of the mitochondrial membrane permeability transition (MPT). As a result, mitochondria swell, leading to the rupture of their outer membrane and the release of cytochrome *c* from the intermembrane space, which is necessary for the induction of apoptosis [6, 7].

Data demonstrating the decrease in the mitochondrial membrane potential are generally obtained using flow cytometry, which does not allow determining why such a decrease occurs. It remains unclear if the decrease is due to the MPT or is mediated by other events, like inhibition of mitochondrial respiration or the uncoupling of oxidative phosphorylation. Besides, it is known that the opening of the pore in the mitochondrial membrane underlies necrotic changes induced by oxidative stress, ischemia and subsequent reperfusion, and disruption of Ca^{2+} homeostasis in the cytoplasm [8].

It is well established that Ca^{2+} is a necessary component of MPT induction. The amount of Ca^{2+} that is necessary for pore opening depends on the presence of agents stimulating the permeability transition, such as inorganic phosphate or oxygen radicals [9]. Pore opening during apoptosis (even in a sub-population of mitochondria) will impair the ability of the entire mitochondrial population to accumulate Ca^{2+}; therefore, estimation of a threshold level of Ca^{2+} loading (the Ca^{2+} capacity of mitochondria) in cells might be a criterion of the intactness of mitochondria. To elucidate whether mitochondria in apoptotic cells undergo permeability transition, we studied accumulation of Ca^{2+} in thymocyte mitochondria after induction of apoptosis by dexamethasone.

A decrease in the mitochondrial membrane potential in thymocytes was observed soon after dexamethasone treatment [6]. It was shown earlier that dexamethasone induces an increase in the Ca^{2+} concentration in the cytoplasm [10], but other observations point to the redistribution of Ca^{2+} between intracellular pools [11], which, in the case of accumulation of Ca^{2+} in mitochondria, might facilitate pore opening.

MATERIALS AND METHODS

Male Wistar rats weighing 90-100 g were used in the experiments. Thymocytes were isolated in RPMI

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media supplemented with 10% fetal serum. Tissue was gently passed through nylon gauze [12], and the cells were washed two times and resuspended to $5 \cdot 10^6$ cells per ml. Apoptosis was initiated by addition of 1 μM dexamethasone. DNA fragmentation was assessed by electrophoresis in agarose gel [13]. For DNA electrophoresis, the cells were lysed in 1% SDS. Then 1 ml 4 M NaCl was added and samples were twice deproteinized with an equal volume of chloroform–isoamyl alcohol mixture (24 : 1 v/v). The aqueous phase was supplemented with two volumes of ethanol and left overnight at –24°C. After precipitation, the DNA was washed with 70% ethanol, dried in air, and dissolved in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The concentration of DNA was determined at 260 nm. DNA electrophoresis (2 h, 80 V) was performed in 1.8% agarose gel and visualized by UV illumination after ethidium bromide staining (1 μg/ml).

The accumulation of Ca^{2+} by mitochondria in digitonin-permeabilized thymocytes [14] was assessed using a Ca^{2+}-sensitive electrode. Thymocytes (10^8 cells) were resuspended in a buffer containing 0.1 M KCl, 1 mM KH_2PO_4, 3 mM $MgSO_4$, 5 mM succinate, and 5 mM Tris, pH 7.4. After 1 min cells were permeabilized with 0.005% digitonin, and 5 μM rotenone was added. Permeabilization with digitonin allows mitochondria to accumulate Ca^{2+} in the presence of a respiratory substrate. Mitochondria were loaded with Ca^{2+} until the release of this cation from mitochondria occurred. The threshold value of Ca^{2+} loading was estimated by summation of the amount of Ca^{2+} in each addition.

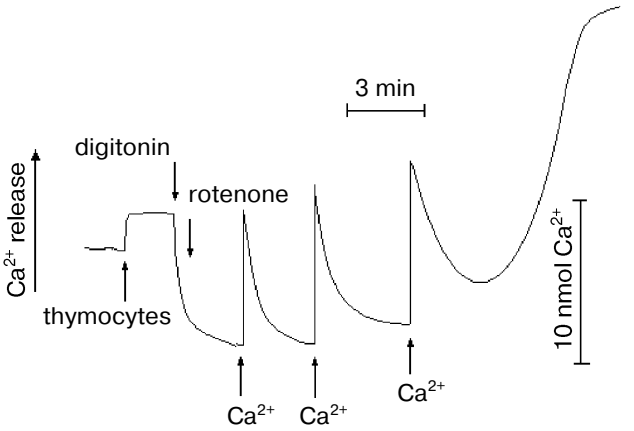

Fig. 1. Estimation of the Ca^{2+} capacity of mitochondria. The plasma membrane of thymocytes (10^8 cells) was permeabilized with 0.005% digitonin and 5 μM rotenone was added. MPT was induced by sequential additions of Ca^{2+}. Ca^{2+} fluxes were monitored with a Ca^{2+}-selective electrode in a buffer containing 0.1 M KCl, 1 mM KH_2PO_4, 3 mM $MgSO_4$, 5 mM succinate, and 5 mM Tris, pH 7.4.

RESULTS AND DISCUSSION

Accumulation of Ca^{2+} in thymocyte mitochondria after plasma membrane permeabilization with digitonin leads to the opening of a pore that is sensitive to cyclosporin A [15]. As seen in Fig. 1, mitochondria accumulate sequential additions of Ca^{2+} that results in a subsequent spontaneous release of the accumulated cations from the mitochondria.

The estimation of the Ca^{2+} capacity of mitochondria in control thymocytes and thymocytes undergoing dexamethasone-induced apoptosis after 4-h incubation did not reveal significant changes (Fig. 2, *2* and *3*). It should be mentioned, however, that in both cases the Ca^{2+} capacity of the mitochondria was lower than that in thymocytes immediately after isolation (Fig. 2, *1*). It is known that the induction of MPT can be prevented by cyclosporin A [15, 16]. Under the conditions of our experiments, addition of 1 μM cyclosporin A together with dexamethasone (or to control thymocytes) completely prevented the decrease in mitochondrial Ca^{2+} capacity of apoptotic thymocytes (Fig. 2, *4*) as well as in control (data not shown).

To evaluate the significance of mitochondrial changes in programmed cell death, mitochondrial Ca^{2+} capacity and the extent of DNA internucleosomal fragmentation was compared. The internucleosomal fragmentation of DNA with a specific "laddering" is one of the most characteristic features of dexamethasone-induced apoptosis in thymocytes [17, 18]. Incubation of

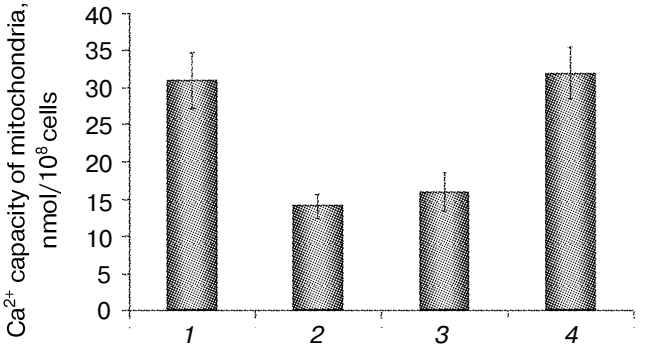

Fig. 2. The Ca^{2+} capacity of mitochondria of thymocytes without incubation (*1*), after 4-h incubation (*2*), after 4-h incubation with dexamethasone (*3*), and after 4-h incubation with dexamethasone and cyclosporin A (*4*). The Ca^{2+} capacity was estimated in buffer containing 0.1 M KCl, 1 mM KH_2PO_4, 3 mM $MgSO_4$, 5 mM succinate, and 5 mM Tris, pH 7.4. Concentrations of dexamethasone and cyclosporin A, 1 μM.

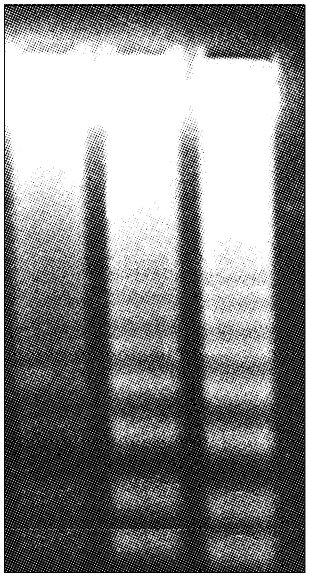

Fig. 3. Internucleosomal DNA fragmentation. Lanes: *1*) after 4-h incubation; *2*) after 4-h incubation with dexamethasone (1 μM); *3*) after 4-h incubation with dexamethasone (1 μM) and cyclosporin A (1 μM).

thymocytes for 4 h lead to spontaneous internucleosomal fragmentation of DNA (Fig. 3, lane *1*) due to the presence of different subpopulations of thymocytes, in particular, those entering the apoptosis phase, as well as preapoptotic cells [19]. Dexamethasone markedly stimulated DNA fragmentation (Fig. 3, lane *2*). Cyclosporin A did not prevent internucleosomal fragmentation of DNA (Fig. 3, lane *3*) and did not stimulate it by itself in control thymocytes (data not shown).

It follows from the data presented that there is no correlation between internucleosomal DNA fragmentation and the sensitivity of mitochondria to MPT induction. The decrease in Ca^{2+} capacity was observed in both control and dexamethasone-treated thymocytes, but the most prominent DNA fragmentation occurred only in the presence of dexamethasone. Incubation with cyclosporin A completely prevented the decrease in the Ca^{2+} capacity but failed to prevent DNA fragmentation. The lack of the sensitivity to cyclosporin A means that the fragmentation of DNA is not mediated by the opening of the pore. This result correlates well with data showing that during apoptosis mitochondria remain functionally active even when substantial changes in cell morphology or typical DNA fragmentation were observed [20].

The assumption that mitochondria in apoptotic cells undergo permeability transition is needed to explain the mechanism of cytochrome *c* release that is necessary for the induction of apoptosis [7], since mitochondrial swelling leads to subsequent rupture of the outer mitochondrial membrane. However, in some publications the pivotal role of mitochondria in apoptosis initiation is questioned [21]. According to data obtained using the fluorescent dye JC-1, mitochondria are functionally intact during the early phases of apoptosis, when DNA fragmentation occurs, whereas changes in their potential takes place after DNA damage [22]. According to the authors' viewpoint, early changes in mitochondria in apoptotic cells were observed because the use of the fluorescent dye DioC-6 as an indicator for mitochondrial membrane potential, which is less sensitive and less specific than JC-1.

As shown in recent publications [23, 24], in some cases during apoptosis cytochrome *c* can be released from mitochondria without MPT induction. The mechanism of cytochrome *c* release from the intermembrane space of mitochondria is not clear yet, but it is supposed to occur through channels formed in the outer mitochondrial membrane by Bcl-2 family proteins such as Bax, Bad, or Bid [25, 26].

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