Key Role of Mitochondria in Apoptosis of Lymphocytes

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Changes in the mitochondrial potential, expression of phosphatidylserine, parameters of direct and lateral light scattering, and DNA fragmentation during spontaneous and induced apoptosis in peripheral blood lymphocytes were studied by flow cytofluorometry. Dexamethasone and Ca^{2+} ionophore A23187 served as inductors of apoptosis. A decrease in the mitochondrial potential is an early sign of spontaneous and induced apoptosis. Phosphatidylserine expression on the outer plasma membrane occurred later and inversely depended on the mitochondrial potential. Our results indicate that the involvement of mitochondria in spontaneous and induced apoptosis accompanied by a decrease in the mitochondrial potential is an early and key event of programmed lymphocyte death. The decrease in the mitochondrial potential of lymphocytes induced degradation of their nuclei (DNA fragmentation) and promoted elimination of apoptotic cells (phosphatidylserine expression).

Key Words: *apoptosis; lymphocyte; mitochondrial potential; phosphatidylserine; DNA fragmentation*

Apoptosis is characterized by various morphological and biochemical changes in nuclei, including chromatin condensation, karyopyknosis, and DNA fragmentation. Programmed cell death is also accompanied by changes in the cytoplasm (reduction of mitochondrial transmembrane potential, condensation and shrinking of granules, widening of the endoplasmic reticulum, *etc*.) and cell membrane (loss of villi, formation of swollen vesicles, appearance of phosphatidylserine and thrombospondin molecules on the cell surface, and increase in membrane permeability for small molecules, including propidium iodide). Changes in the nucleus are not the only manifestations of apoptosis. Cytoplasmic structures, including mitochondria, act as regulators of apoptosis. Enucleated cells can also undergo apoptosis, which confirms the notion that nuclei are not the main regulator this process. Published data indicate that changes in the nucleus corresponding to the final and irreversible stage of apoptosis (*e.g.*, DNA fragmentation) should be taken into account when studying this process. However, the absence of these changes does not prevent programmed cell death.

Various factors are involved in the regulation of apoptosis. In this respect, much attention is given to mitochondria. Previous studies showed that isolated mitochondria induce disintegration of nuclei in cell-free cultures, which is related to the presence of biologically active substances in the matrix and intermembrane space of these organelles (apoptosis-inducing factor, cytochrome *c*, and procaspases 2, 3, and 9, *etc.*). These factors directly or indirectly (through activation of executing caspases) induce changes typical of apoptosis, including DNA degradation (final and irreversible stage of apoptosis) [8,13]. Various factors are released from mitochondria after opening of mitochondrial membrane channels, which decreases the mitochondrial potential (MP). Thus, the decrease in MP indicates the release of these factors into the cytoplasm and involvement of mitochondria into nucleus degradation.

Expression of specific molecules (*e.g.*, phosphatidylserine, PS) on the cell surface accompanies apoptosis and underlies elimination of apoptotic cells from the body. Thus, the decrease in MP and expression of PS on the cell surface are the very early signs

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of apoptosis. The sequence of these events during apoptosis and dependence of PS expression on MP remain unclear. Despite ample data on the involvement of mitochondria in apoptosis, their priority [1,2,4,12,14] and necessity [5,6,9] for this process are still controversial.

Here we studied the sequence of events during spontaneous and induced apoptosis in lymphocytes and their dependence on MP, which characterizes the release of various apoptogenic factors from mitochondria.

MATERIALS AND METHODS

Peripheral blood lymphocytes were obtained from 28 healthy donors. The cells were isolated by centrifugation in a Percoll density gradient (Pharmacia). Erythrocytes were precipitated with 4.5% dextran T-500 (Loba Chemie). The cells isolated from interphase 1.077 were cultured in 96-well flat-bottom plastic plates (Nunc) in RPMI-1640 medium (Flow) containing 10% fetal bovine serum, 200 μg/ml L-glutamine (Flow), and antibiotics. These cells were incubated with dexamethasone (10^{-4}-10^{-6} M), Ca^{2+} ionophore A23187 (1-5 μM), oligomycin (100 μM), and m-ClCCP (50-150 μM) in a CO_2 incubator for 144 h. All reagents were from Sigma. Samples were taken at 24-h intervals.

Apoptosis in lymphocytes was studied by flow cytofluorometry in a Facscan device (Becton Dickinson). DNA fragmentation was evaluated by staining with hypotonic propidium iodide (Sigma) containing 0.1% Triton X-100 and 0.1% sodium citrate. The cells were fixed with 70% ethanol before staining. The percentage of apoptotic cells in the hypodiploid histogram zone (to the left from the major peak corresponding to diploid cells) was evaluated. The results were recorded on a secondary fluorescence detector (Fl2). MP was estimated after straining with fluorochromes CMXRos (Molecular Probes) and $DiOC_6$ (Sigma). The results were recorded on secondary (Fl2) and primary fluorescence detectors (Fl1), respectively. In some experiments fluoresce of fluorochromes used for estimating MP was controlled by incubating lymphocytes with protonophore m-ClCCP for 15 min. This agent selectively decreases MP. PS expression was estimated with fluorochrome MC540 (Sigma). The results were recorded on a secondary fluorescence detector (Fl2).

Changes in direct (DLS) and lateral light scattering (LLS) were evaluated by a shift in conventional lymphocyte gate.

RESULTS

Culturing of cells in various media, including RPMI-1640, caused their death, in particular, via apoptosis. Initiation of programmed cell death during their culturing was associated with a deficiency of growth factors specific for each type of cells. Growth factors added to the culture medium considerably increased cell lifespan. The addition of interleukin-2 to the culture medium at 1-day intervals by several times increased lymphocytes lifespan. Thus, culturing of lymphocytes in a growth factor-free medium is an appropriate model for studying apoptosis.

In our experiments incubation of lymphocytes in RPMI-1640 medium caused their death (necrosis or apoptosis). Lymphocyte death was accompanied by changes in the cytoplasm, nucleus, and cell surface. Cytoplasmic changes included MP reduction after 48-h incubation. The intensity of MC540 fluorescence on lymphocytes increased, which indicated expression of PS on their surface. In the next stages (72 h and later) changes in DLC and LLS were accompanied by a decrease in MP and intensive expression of PS. DNA fragmentation was also initiated during this period.

Thus, the very early signs of spontaneous apoptosis in lymphocytes include changes in MP and expression of PS on the cell surface. These parameters underwent variations even in cells with unchanged light scattering. MP progressively decreased, while the intensity of PS expression increased in the gate for lymphocytes with altered optical parameters. These results indicate that the decrease in MP during apoptosis in lymphocytes precedes, but not coincides with their shrinkage [2].

After incubation in RPMI-1640 medium for 48 and 72 h the count of lymphocytes with lowered MP surpassed the number of cells expressing PS. The counts of PS-positive cells with low MP were practically similar after 96-h incubation. We hypothesized that the MP decrease is inversely related to the intensity of PS expression. This assumption was confirmed after simultaneous staining of lymphocytes with $DiOC_6$ and MC540 reflecting MP and PS expression, respectively. PS was expressed only on cells with reduced MP. Lymphocytes with unchanged MP did not express PS on their surface.

Opening of mitochondrial membrane channels leads to the release of various apoptogenic factors from the intermembrane space. This process is accompanied by a decrease in MP. Various factors, including reactive oxygen species, high intracellular Ca^{2+} content, imbalance between intracellular ADP and ATP, and exhaustion of glutathione and NADPH reserves, stimulate opening of mitochondrial membrane channels. Thus, opening of channels should be followed by a decrease in MP and induction of PS expression on lymphocyte surface.

Incubation of lymphocytes with protonophore m-ClCCP for 30 min induced a dose-depend de-

crease in MP. PS was expressed on lymphocyte surface at later terms of incubation. A negative correlation was found between changes in PS expression and MP.

Oligomycin-induced blockade of mitochondrial ATPase led to a decrease in MP and promoted PS expression on the cell surface. MP in lymphocytes decreased after 72-h incubation, while PS expression was observed at later terms of incubation and only on cells with low MP.

The increase in intracellular Ca^{2+} content induced by calcium ionophore A23187 was followed by dose-dependent changes in test parameters. A23187 in high doses (5 μM) decreased MP after 30-min incubation, while in low doses this preparation produced similar changes after incubation for 1 and 24 h (2 and 1 μM, respectively). During A23187-induced apoptosis expression of PS on lymphocyte surface followed the decrease in MP and was inversely related to this parameter.

Glucocorticoids are inductors of apoptosis in lymphoid cells (*e.g.*, thymocytes). MP decreased after 24-h incubation of lymphocytes with dexamethasone. However, the count of cells expressing PS increased only after 48-72-h incubation.

Thus, the decrease in MP is the very early sign of programmed cell death that precedes changes in the cell volume and expression of PS. Moreover, the decrease in MP induces PS expression on the cell surface.

Incubation of lymphocytes led to DNA fragmentation, which resulted from caspase activation. This process followed a decrease in MP, appearance of PS on the cell surface, and changes in DLS and LLS. The sequence of these events in lymphocytes was similar after incubation with mitochondrial ATPase inhibitor oligomycin, Ca^{2+} ionophore A23187, and dexamethasone.

Thus, during spontaneous and induced apoptosis DNA fragmentation is a final event that probably occurs only in lymphocytes with reduced MP. It is impossible to evaluate changes in both parameters, since fixation of cells during DNA staining is followed by a marked decrease in MP. The decrease in MP and DNA fragmentation are accompanied by changes in optical characteristics of cells. Moreover, MP decreases even in optically unchanged cells incubated with Ca^{2+} ionophore and oligomycin. These data suggest that the decrease in MP and DNA fragmentation in lymphocytes have the same directionality.

Our results indicate that the decrease in lymphocyte MP is the very early sign of spontaneous and induced apoptosis. The decrease in MP indicates the release of various apoptogenic factors from mitochondria, which is necessary for further changes in the nucleus and plasma membrane. The sequence of intracellular events observed after incubation with Ca^{2+} ionophore and dexamethasone indicates that mitochondria are universal integrators of apoptogenic stimuli. The release of biologically active substances from these organelles contributes to PS expression on the cell surface, cell shrinkage, DNA degradation, and other signs of apoptosis.

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