GENERAL PATHOLOGY AND PATHOPHYSIOLOGY

Modulation of Apoptosis of Mononuclear Cells under Conditions of Oxidative Stress

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We studied mitochondrial and type 1 tumor necrosis factor-α receptor (TNFR1)-mediated pathways triggering the apoptotic program in mononuclear cells under conditions of oxidative stress. Intensification of intracellular production of reactive oxygen forms is accompanied by an increase in the number of annexin-positive TNFR1-presenting cells and mononuclear cells with reduced mitochondrial transmembrane potential in case of induction of oxidative stress with 1 mM H_2O_2 *in vitro* and in patients with pneumonia.

Key Words: *oxidative stress; reactive oxygen forms; apoptosis; mitochondrion; receptor*

Reactive oxygen species (ROS), essential attributes of vital activity of all aerobic organisms, ensure adaptive reaction of cells to external stimuli. However, there is no clear-cut boundary between physiological and pathological functions of these molecules [4]. For instance, ROS act as second messengers mediating the effect of ligand-receptor interactions. Among these ligands are hormones, cytokines, and growth factors. In this case, ROS actively participate in signal transduction and modulate the key reactions of metabolic pathways in cells (phosphorylation, Ca^{2+} metabolism, hydrolysis of phospholipids, modulation of transcription factors, *etc*.) [5,6].

Various stress factors induce cell response aimed a mobilization of resources for preventing negative consequences of stress. ROS participate in signal transduction, expression of some genes in adaptive reaction of cells (*e.g*. in cell proliferation and differentiation) under extreme conditions [4]. Exhaustion of adaptation resources against the background of prolonged stress leads to depletion of antioxidant defense reserves: the rate of ROS generation exceeds the rate of their detoxification. This state can be characterized as oxidative stress (OS) [6]. High concentrations of ROS initiate necrotic or apoptotic cell death. Realization of this or that effect depends on the strength and nature of the initiating stimulus, cell sensitivity to OS, state of the antioxidant defense system, and activity of repair systems [5].

Intensification of oxidative reactions under pathological conditions can affect apoptosis (both its activation and inhibition), thus acting as an additional pathogenetic mechanism in the development of cardiovascular and oncological diseases, inflammatory and neurodegenerative processes [3]. Of particular interest is the role of changes in the redox state of the cell in apoptosis modulation [9]. At the same time, peculiarities of functioning of

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individual systems of transmission of apoptotic signals (extra- and intracellular) under conditions of changed oxidative metabolism require detailed investigation.

Here we determined the role of receptor-mediated and mitochondrial pathways in triggering of apoptosis under conditions of OS.

MATERIALS AND METHODS

Peripheral blood was obtained from 20 healthy donors and 18 patients with documented community-acquired pneumonia. Mononuclear leukocytes (MNL) were isolated from the blood by centrifugation in Ficoll density gradient (ρ=1.077) and resuspended in complete nutrient medium: 90% RPMI-1640, 10% heat-inactivated (30 min at 56°C) FCS (Biolot), 0.3 mg/ml L-glutamine, 100 μg/ml gentamicin, and 2 mM/ml HEPES (Flow). MNL were incubated for 18 h at 37°C and 5% CO_2. For induction of OS, H_2O_2 in concentrations of 1, 500, 100, 50, and 10 μM was added to the cell cultures.

The intensity of ROS generation in cells and activity of apoptosis in MNL culture were evaluated by the method of laser flow cytometry (Epics XL, Beckman Coulter) using dichlorofluorescin diacetate (DCF-DA, Sigma Aldrich) and FITC-labeled annexin V (Catlag), respectively. The parameter characterizing ROS content in MNL was expressed in arbitrary units (fluorescence intensity per cell).

The content of cells with low mitochondrial transmembrane potential was determined using Mito-Screen kits (BD Pharmigen). The method explores fluorochrome JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbocyanine iodide) existing in two different states (aggregates and monomers) characterized by different fluorescence spectra in FL-1 and FL-2 channels of the flow cytometer. MLN suspension (1 ml, 10^6 cells) was centrifuged at 1000 rpm for 5 min at room temperature, the cells were resuspended, incubated with 0.5 ml fresh JC-1 solution (prepared according to manufacturer's instructions) at 37°C for 10-15 min, and then washed twice. JC-1-stained lymphocytes were analyzed on flow cytometer Epics XL (Beckman Coulter).

For counting cells presenting type 1 membrane receptors to TNF-α (TNFR1, CD120), MNL were washed with phosphate buffer (pH 7.2) and stained with standard monoclonal FITC-labeled antibodies to this receptor (Immunotech) according to manufacturer's instructions. Analysis of samples was performed on a flow cytometer. Green fluorescence from FITC was analyzed on a one-dimensional histogram in lymphocyte and monocyte gates.

The concentration of TNF-α in supernatants of MNL culture was determined by ELISA on a Multiscan EX reader (ThermoLabSistems) using Procon kits according to manufacturer's instructions.

The data were processed by methods of variation statistics. Normal distribution of the obtained results was verified using Kolmogorov—Smirnov test. Reliability of differences was evaluated using nonparametric Mann—Whitney (for independent samples) and Wilcoxon (for dependent samples) tests. The differences were significant at $p<0.05$. The data are presented as median (Me) and upper and lower quartiles (Q_1-Q_3).

RESULTS

Oxidative metabolism imbalance is associated with the development of various pathologies, including inflammatory processes of different etiology [4]. This is confirmed by increased (compared to normal) content of ROS in MNL of patients with acute pneumonia comparable to that in case of experimentally induced OS (Table 1, 2).

Considerably increased count (compared to normal) of annexin-positive cells was found in MNL culture from healthy individuals after *in vitro* induction of OS (1 mM H_2O_2) and in MNL culture from patients with pneumonia (Table. 2).

Induction of apoptosis can be related to activation of both extra- and intracellular pathways. Among intracellular pathways, the mitochondrial pathway plays an important role, because of the presence of abundant proapoptotic factors (cytochrome C, Smac, AIF, endonuclease G) in mitochondria [8, 12]. The release of these factors from the intermembrane space into cytosol activates procaspase-9, thus triggering apoptosis [1]. This process is impossible without opening of permeability transition pores between the outer and inner mitochondrial membranes and formation of channels in the outer membrane, which leads to a drop of transmembrane potential [12]. The decrease of mitochondrial transmembrane potential was recorded only in cells exposed to 1 mM H_2O_2 and in cells from patients with pneumonia (Table 2). It is known that mitochondria are the main target and the main source of ROS in cells. They change their functional characteristics (including $\Delta\psi$) depending on intracellular content of antioxidants [4]. It can be hypothesized that redox-dependent decrease in $\Delta\psi$ is an element of activation of apoptogenic mechanisms observed during OS.

Receptor-mediated pathway (for instance, TNF-mediated) plays an important role in initiation of apoptosis. It implies binding of TNFR1 with the

TABLE 1. Content of ROS, Number of Cells wit Reduced Transmembrane Potential, and Count of Apoptotic Cells in Total MNL Population from Healthy Donors under Conditions of OS ($Me(Q_1-Q_3)$)

Experimental conditions	ROS content in cell, arb. units	Number of cells with reduced transmembrane potential, %	Count of annexin-positive cells, %
Intact cells	0.28 (0.19-0.35)	1.35 (1.10-1.65)	1.57 (1.02-2.14)
Incubation with H_2O_2			
10 μM	0.15 (0.09-0.23)	1.63 (1.18-1.93)	1.34 (0.96-1.82)
50 μM	0.14 (0.12-0.23)	1.73 (1.33-2.61)	1.68 (1.15-2.21)
100 μM	0.21 (0.18-0.29)	1.96 (0.80-3.18)	1.39 (0.26-4.97)
500 μM	0.32 (0.27-0.33)	1.32 (0.76-2.44)	1.30 (0.51-2.06)
1 mM	0.55 (0.49-0.63)*	9.07 (6.17-9.27)*	11.08 (10.42-14.62)*

Note. Here and in Table 2: *$p<0.001$ compared to intact cells.

TABLE 2. Intracellular Production of ROS, Content of TNF-α in Supernatants, Content of Apoptotic and TNFR1-Presenting Cells, and MNL with Reduced Transmembrane Potential in General Population of MNL from Peripheral Blood of Healthy Donors under Conditions of OS *in Vitro* and in Patients with Pneumonia ($Me(Q_1-Q_3)$)

Experimental conditions	Intact MNL from healthy donors	Incubation of MNL from healthy donors with 1 mM H_2O_2	Intact MNL from patients with pneumonia
ROS content in cell, arb. units	0.28 (0.19-0.35)	0.55 (0.49-0.63)*	0.49 (0.41-0.58)*
Count of annexin-positive cells, %	1.57 (1.02-2.14)	9.07 (6.17-9.27)*	10.14 (9.89-13.57)*
Number of cells with reduced Δψ, %	1.35 (1.11-1.65)	9.07 (6.17-9.27)*	7.54 (5.02-8.86)*
Number of TNFR1-presenting cells, %	1.45 (0.96-2.05)	8.89 (5.83-10.49)*	10.21 (9.15-1.97)*
Content of TNF-α in supernatants	96 (88-107)	105 (99-117)	150 (139-166)*

corresponding ligand and formation of a complex between intracellular areas of the receptor and adaptor protein TRADD (TNFR-associated death domain). The latter induces oligomerization of caspase-8 and its autoproteolysis [1,2,7]. Caspase-8 affects effector proteases (caspases 3 and 7), which activate caspase 6 cleaving nuclear mitotic apparatus protein and mediating shrinkage and fragmentation of the nucleus [10].

Comparison of the count of TNFR1-carrying cells revealed an increase in this parameter from 1.45% (normal 0.96-2.05%) to 10.21% (9.15-10.97%) in MNL from patients with pneumonia and to 8.89% (5.83-10.49%) in cell culture after OS induction *in vitro* (Table 2). The increase in the number of TNFR1-presenting cells in the culture was associated with enhanced apoptosis against the background of intensified ROS generation. It can be hypothesized that imbalance of oxidative metabolism modulates the function of signaling systems controlling the processes related to expression and presentation of TNFR1 and regulation of apoptosis. Indeed, ROS can affect cell viability by modulating the expression of some genes via activation of the corresponding transcription factors (p53, NF-κB, and AP1) [5]. It was found that p53 can increase expression of TNFR, thus sensitizing the cells to the corresponding ligand [14], whose binding activates the intracellular signal cascade. In turn, synthesis of TNF-α is controlled by redox-sensitive transcription factor NF-κB [13]. However, our findings showed that incubation with H_2O_2 (1 mM) did not modulate production of TNF-α by MNL obtained from healthy donors, probably due to posttranslation modification of NF-κB leading to changes in the spectrum of genes induces by this cytokine [11]. At the same time, we observed enhanced production of TNF-ligand by MNL from patients with acute inflammation compared to the control. Under conditions of inflammation, synthesis of TNF-α in the organism is induced mainly by bacterial lipopolysaccharides and cytokines IL-1-, IL-2, IFN-γ, and granulocyte-macrophage CSF [11].

Our findings attest to dysregulation of TNF-mediated pathway of apoptosis induction in MNL, because enhanced production of TNF-α during acute inflammation did not affect changes in their apoptotic activity compared to that in experimental OS.

This effect can be explained by switching over (at the level of TRADD adapter protein) the apoptotic signal to activating one participating in the induction of NF-κB via special kinase [13].

Thus, the mechanisms of modulation of apoptosis under conditions of oxidative metabolism imbalance involve mitochondrial and TNFR-mediated pathways.

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