Role of Reactive Oxygen Species and Bcl-2 Family Proteins in TNF-α-Induced Apoptosis of Lymphocytes

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We studied the *in vitro* apoptosis-inducing effect of recombinant TNF-α (rTNF-α) on blood lymphocytes from healthy donors. rTNF-α-induced apoptosis was accompanied by an increase in the number of cells with low mitochondrial transmembrane potential, increased intracellular content of reactive oxygen species, reduced content of Bcl-2, Bcl-xL, and Bax proteins, and elevated Bad content. The molecular mechanisms of these changes are discussed.

Key Words: *tumor necrosis factor-α; apoptosis; mitochondria; reactive oxygen species; Bcl-2 family proteins*

Apoptosis is a genetically determined process aimed at maintenance of cell homeostasis in the body. Among a variety of factors regulating apoptotic death of immunocompetent cells, TNF-α plays a particular role in this process. TNF-α binds to complementary receptors on the cell surface and induces the tanatogenic program [10]. However, TNF receptor can also transfer an antiapoptotic signal or proliferative information. It depends on the dose of this cytokine, functional activity of cells, state of the microenvironment, and other factors [1].

TNF-α initiates the extrinsic apoptotic pathway, which is related to activation of the caspase cascade. An inducing agent, caspase-8, can also transmit the apoptotic signal in mitochondria [10]. Studying the apoptotic role of these organelles revealed the existence of two interrelated mechanisms. They are coupled to the generation of reactive oxygen species (ROS) and Bcl-2 protein-regulated compartmentalization of apoptosis-inducing agents in the intermembrane mitochondrial space [2,5]. Little is known about the role of these processes in NTF-α-induced apoptosis [10]. Here we evaluated the role of ROS and Bcl-2 family proteins in TNF-α-induced apoptosis of blood lymphocytes.

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MATERIALS AND METHODS

In vitro experiments were performed on lymphocytes from 12 healthy donors (5 men and 7 women, 22-30 years). These cells were isolated from the venous blood on a Ficoll-Paque density gradient (Pharmacia, ρ=1.077 g/cm^3). The cells were cultured in RPMI-1640 medium with 10% FBS and 0.03 mg/ml L-glutamine at 37°C and 5% CO_2 for 18 h. To evaluate the mechanisms of TNF-α-induced apoptosis, recombinant human TNF-α (rTNF-α, Biosource) in an apoptogenic concentration of 0.050 ng/ml was added to the parallel sample. The number of apoptotic cells (annexin-positive) and necrotic cells (propidium iodide staining) was estimated on an Epics XL flow cytofluorometer (Beckman Coulter) using FITC-labeled annexin V (Beckman Coulter) and propidium iodide (Beckman Coulter) [12].

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TABLE 1. Percent of Annexin-Positive Lymphocytes, Percentage of Cells with Low Mitochondrial Potential, and Intracellular ROS Concentration during Incubation with rTNF-α in an Apoptosis-Inducing Dose (*Me* (Q_1-Q_3)

Parameter	Intact culture of lymphocytes	Incubation of lymphocytes with rTNF-α
Ratio of annexin-positive cells, %	1.69 (1.04-2.08)	8.73* (7.30-12.40)
Ratio of cells with low transmembrane potential of mitochondria, %	2.24 (1.34-2.89)	4.54* (1.76-6.78)
Intracellular ROS concentration, rel. units	18 (13-19)	79* (76-326)

Note. Here and in Table 2: *p<0.05 compared to intact culture.

The decrease in transmembrane mitochondrial potential was recorded cytofluorometrically with MitoScreen kit (BD Pharmigen). A key reagent JC-1 is present in viable cells in the form of monomers and aggregates (green and light fluorescence, respectively). During the impairment of mitochondrial membrane integrity, JC-1 cannot form the aggregates (green-stained cells) [13].

The intracellular concentration of ROS was estimated with non-fluorescent dichlorofluorescin diacetate (Sigma Aldrich). The degree of cell fluorescence was evaluated. The concentration of ROS in lymphocytes was calculated as the ratio of cell fluorescence to lymphocyte number (multiplied by 1000) and expressed in relative units.

The content of Bcl-2 family proteins (Bax, Bad, Bcl-2, and Bcl-xL) in lymphocyte lysates was measured by the Western blotting method. The cell culture was treated with a lysing buffer (50 mM Tris-HCl, pH 6.5), 100 mM dithiothreitol (Helicon), 2% dimethylsulfoxide (Helicon), 0.1% bromophenol blue (Helicon), 15% glycerol (Helicon), 0.02% β-mercaptoethanol (Helicon), and mixture of proteinase inhibitors (Sigma Aldrich). The proteins were separated electrophoretically in 5 and 10% sodium dodecyl sulfate-PAAG and transferred to a Bio-Rad nitrocellulose membrane. The membranes were subsequently incubated in phosphate buffered saline with 0.05% Tween 20, 5% defatted dry milk, and primH,y antibodies to Bax, Bad, Bcl-2, and Bcl-xL proteins (Sigma Aldrich). The membrane was treated with peroxidase-labeled secondary antibodies (Biosource) and horseradish peroxidase substrate (from tetramethylbenzidine). The enzyme G3PDH (Chemicon) was used as an internal standard. The data were expressed in arbitrary units.

The results were analyzed statistically. The normality of distribution was evaluated by Kolmogorov–Smirnov test. The median (Me) and upper and lower quartiles (Q_1 and Q_3) were calculated for each sample. The equality of the means was estimated by Mann–Whitney *U* test. The differences were significant at p<0.05.

RESULTS

Incubation of lymphocytes in the medium with rTNF-α at an apoptosis-inducing dose was accompanied by a significant increase in the number of annexin-positive cells (as compared to the intact culture; Table 1). TNF-α serves as one of the standard inductors of apoptosis, which is realized via the receptor pathway. It suggests the recruitment of adapter molecules TRADD and FADD that are associated with the TNF-α type I receptor (TNF-RI). These changes result in activation of caspase-8 [10]. One the one hand, caspase-8 induces the apoptotic caspase cascade. On the other hand, this enzyme has a direct effect on mitochondria through the protein Bid. It contributes to the formation of pores and membrane depolarization in organelles [9]. Taking into account these data, we evaluated the number of cells with low transmembrane potential of mitochondria. Treatment with rTNF-α in a programmed cell death-inducing dose was followed by an increase in the number of these cells (Table 1).

Mitochondria are the major intracellular producers of oxidative equivalents (ROS). Therefore, structural changes in the membrane of these organelles are always accompanied by variations in intracellular ROS. The exposure of lymphocytes to rTNF-α was followed by ROS accumulation (Table 1). It is probably related to the fact that TNF-α induces the production of ROS in the mitochondrial electron transport chain [14].

The imbalance in cellular redox status due to the excessive content of ROS can be followed by dysfunction of various components in cell signaling. Mitogen-activated protein kinases (MAP kinases) are highly sensitive to changes in the redox state [3,4]. For example, ROS accumulation leads to the release of enzyme ASK1 kinase from the complex with its specific inhibitor [2]. JNK kinase is induced after activation of ASK1 kinase. This enzyme is involved in the realization of TNF-α-induced apoptosis. Experiments on cultured HeLa cells showed that TNF-α induces apoptosis due to stimulation of JNK kinase, which contributes to

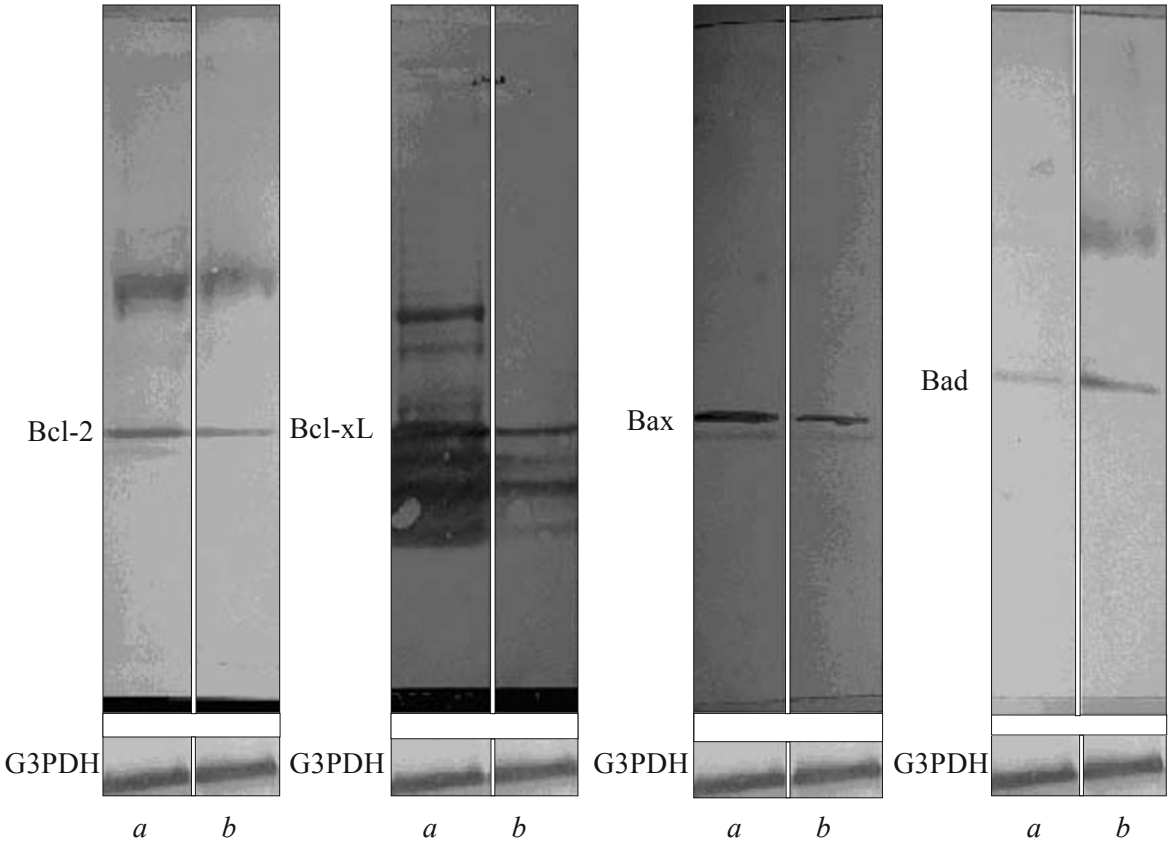

Fig. 1. Content of Bcl-2, Bcl-xL, Bax, and Bad proteins in blood lymphocytes. Intact culture (*a*); incubation of cells in the medium with rTNF-α (*b*).

the release of mitochondrial proapoptotic factors (*e.g.*, cytochrome C, AIF, and HtrA2/Omi) [7]. A variety of stimulating agents can induce the release of these factors into the cytoplasm through permeability transition pores in the outer mitochondrial membrane. Bcl-2 family proteins regulate the formation of these structures [5]. We measured the content of antiapoptotic proteins Bcl-2 and Bcl-xL and apoptosis-promoting proteins Bax and Bad in lymphocytes during culturing in the medium with an apoptosis-inducing dose of rTNF-α. Incubation of lymphocytes with rTNF-α in this dose was followed by a decrease in the content of antiapoptotic proteins Bcl-2 and Bcl-xL and proapoptotic protein Bax (Table 2, Fig. 1).

The decrease in intracellular Bcl-2 concentration could be associated with TNF-α-induced oxidative stress. It contributes to inactivation of ERK1/2 MAP kinases that phosphorylate Bcl-2. Structural stability of Bcl-2 is related to phosphorylation in positions -56, -74, and -87 (ERK1/2). The splitting of a phosphate group in these regions is followed by ubiquitin binding and Bcl-2 degradation [8].

Similar changes in the content of Bax and Bcl-2 are probably associated with the formation of a com-

TABLE 2. Content of Bcl-2 Family Proteins in Peripheral Blood Lymphocytes from Healthy Donors after Culturing with rTNF-α in an Apoptosis-Inducing Dose (*Me* (Q_1-Q_3)

Parameter		Intact culture of lymphocytes	Incubation of lymphocytes with rTNF-α
Content of antiapoptotic proteins, arb. units	Bcl-2	1.30 (1.22-1.45)	0.70* (0.57-0.98)
	Bcl-xL	1.90 (1.59-1.98)	1.13* (0.71-1.30)
Content of proapoptotic proteins, arb. units	Bax	0.97 (0.87-1.04)	0.35* (0.33-0.37)
	Bad	0.57 (0.51-0.59)	0.83* (0.69-0.97)

plex, which abolishes the anti-suicidal effect of Bcl-xL [11]. Individual proteins in this heterodimer cannot be identified due to the unavailability of specific epitopes for antibodies and increase in the molecular weight of native proteins after heterodimerization.

The amount of proapoptotic Bad was shown to increase in lymphocytes (Table 2, Fig. 1). These changes could be related to overexpression of the *bad* gene, which is positively regulated by the transcription factor p53. Previous experiments on the culture of A549 cells showed that the increase in the concentration of Bad is required for p53 transport into mitochondria. Association of these proteins in mitochondria contributes to the release of apoptogenic cytochrome C into the cytoplasm [9].

We conclude that mitochondrial factors play a role in the regulation of TNF-α-induced apoptosis in lymphocytes. ROS and proteins of permeability transition pores in the mitochondrial membrane play a key role in this process. Our findings should be taken into account in the development of novel approaches to the correction of disorders that are associated with abnormal production of TNF-α. Such approaches are directed towards the regulation of programmed cell death.

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