IMMUNOLOGY AND MICROBIOLOGY

Role of NF-kB, p53, and p21 in the Regulation of TNF-α Mediated Apoptosis of Lymphocytes

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The effect of recombinant TNF-α on programmed death of donor lymphocytes was studied *in vitro*. The proapoptotic effect of this cytokine is realized through transcription factors and cell cycle inhibitors. Incubation of lymphocytes with recombinant TNF-α revealed increased levels of NF-kB and p21 and reducted content of nonphosphorylated p53.

Key Words: *tumor necrosis factor-α; apoptosis; NF-kB; p53; p21*

TNF-α is a pleiotropic cytokine playing an important role in the regulation of immune response. One of the mechanisms of realization of this function by TNF-α is the maintenance of lymphocyte count by modulation of their apoptotic death program [3]. Regulation of apoptosis at the molecular level is realized at the expense of a variety of factors with stimulatory or inhibitory effects on this process. Proteins with pro- and antiapoptotic function play an important role in the regulation of programmed cell death. The synthesis of these proteins depends on the presence of active transcription factors, such as NF-kB and p53, forming in response to stimuli coming to the cell. In addition, cell cycle delay is required for triggering the apoptosis program. It is determined by accumulation of cyclin-dependent kinase inhibitors, *e.g.* $p21^{WAF1/Cip1}$ [1].

The data on the role of NF-kB, p53, and $p21^{WAF1/Cip1}$ in TNF-α-induced death are contradictory. It was shown on different cell strains that apoptosis developing under the effect of TNF-α is associated with predominant activation of only one of the transcription factors, NF-kB or p53 [5,7,13]. We evaluated the involvement of transcription factors NF-kB and p53 and of $p21^{WAF1/Cip1}$ cyclin-dependent kinase inhibitor into the realization of TNF-α-mediated apoptosis of blood lymphocytes.

MATERIALS AND METHODS

The experiment was carried out on lymphocytes from 12 donors (5 men and 7 women aged 22-30 years). The cells were isolated from venous blood by the standard method on Ficoll-Paque density gradient (Pharmacia; ρ=1.077 g/cm^3) and cultured for 18 h at 37°C and 5% CO_2 in RPMI-1640 with 10% FCS and 0.03 mg/ml L-glutamine. Human recombinant TNF-α (rTNF-α; Biosource) in concentrations of 0.015-0.150 ng/ml was added to parallel samples for studies of the mechanisms of TNF-α-induced apoptosis. The counts of apoptotic (annexin-positive) and necrotic cells absorbing annexin V and propidium iodide were evaluated using FITC-labeled annexin V (Beckman Coulter) and propidium iodide (Beckman Coulter) on an Epics XL flow cytofluorometer (Beckman Coulter) [10].

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The content of NF-kB subunit RelA (p65), nonphosphorylated p53, and p21$^{WAF1/Cip1}$ in lymphocyte lysates was measured by Western blotting. Lyzing buffer (50 mM Tris-HCl, pH 6.5), 100 mM dithiotreitol (Helicon), 2% DMSO (Helicon), 0.1% Bromophenol Blue (Helicon), 15% glycerol (Helicon), 0.02% β-mercaptoethanol (Helicon), and a mixture of proteinase inhibitors (Sigma Aldrich) were added to the cell cultures. The proteins were separated in 5 and 10% sodium dodecylsulfate-PAAG in an electrophoretic chamber (Bio-Rad) and then transferred onto nitrocellulose membrane (Bio-Rad) in a transfer box (Bio-Rad). The membranes were incubated, in succession, in phosphate saline buffer with 0.05% Twin-20 with 5% degreased dry milk, first antibodies to NF-kB (p65 RelA), nonphosphorylated p53, and p21$^{WAF1/Cip1}$ (Sigma Aldrich). Then second antibodies with peroxidase label (Biosource) and tetramethylbenzidene-based substrate for horseradish peroxidase were applied onto the membrane. The resultant blots were digitized on an Epson scanner. The content of the target antigen in the cell was evaluated as the proportion of the target protein signal to the G3PDH enzyme (Chemicon) signal using AlphaView Q software for Western blotting results processing (Alpha Innotech). The results were expressed in arbitrary units.

The data were processed by methods of statistical analysis. The normality of parameter distribution was verified using Kolmogorov—Smirnov test. The median (Me) and the first and third quartiles (Q_1, Q_3) were calculated for each sampling. The equality of selected means was evaluated by Mann–Whitney *U* test. The differences were considered significant at $p<0.05$.

RESULTS

In vitro study showed that the increase in rTNF-α concentration in culture medium is associated with an increase in the counts of apoptotic lymphocytes and necrotic cells. This fact attests to a dose-dependent effect of TNF-α on cell death. The minimum concentration of rTNF-α (0.05 ng/ml) significantly increased the count of apoptotic lymphocytes and had no effect on the percentage of necrotic cells (Fig. 1).

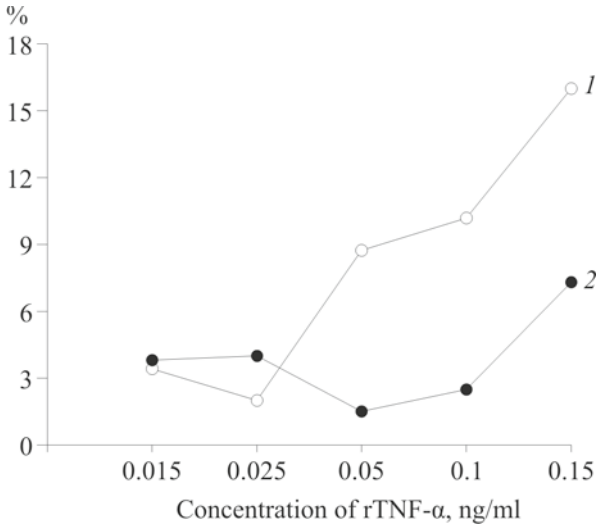

Fig. 1. Changes in the levels of apoptotic (*1*) and necrotic (*2*) cells in blood lymphocyte culture in the presence of different concentration of rTNF-α in the incubation medium

A significant increase in the percentage of annexin-positive lymphocytes after incubation with rTNF-α in comparison with intact culture (Table 1) can be explained by apoptosis triggering by the receptor pathway with possible extra mobilization of the nuclear and mitochondrial proapoptotic factors [8].

Realization of the apoptosis program is preceded by cell cycle arrest mediated through accumulation of cyclin-dependent kinase inhibitors [1]. We therefore evaluated the level of p21$^{WAF1/Cip1}$ and revealed its increase in the presence of rTNF-α (Table 1; Fig. 2). Gene transcription and formation of p21$^{WAF1/Cip1}$ protein product depend on p53 [1]. Activation of p53 in response to various stress factors and cell damage is realized through the formation of the phosphorylated form [4,13]. It is obvious that in our experiments the reduction of nonphosphorylated p53 level in lympho-

TABLE 1. Percentage of Apoptotic Cells, Levels of NF-kB, p53, and p21 in Lymphocytes Cultured with rTNF-α *In Vitro* (Q_1-Q_3)

Parameter	Intact lymphocyte culture	Lymphocytes cultured in medium with rTNF-α
Apoptotic cell %	1.69 (1.04-2.08)	8.73* (7.30-12.40)
Intracellular content of proteins, arb. units		
NF-kB	0.48 (0.36-0.52)	1.02* (0.56-1.08)
nonphosphorylated p53	1.48 (1.42-1.65)	0.69* (0.57-0.99)
p21	0.46 (0.39-0.49)	0.63* (0.55-0.64)

Note. *$p<0.05$ compared to intact lymphocyte culture.

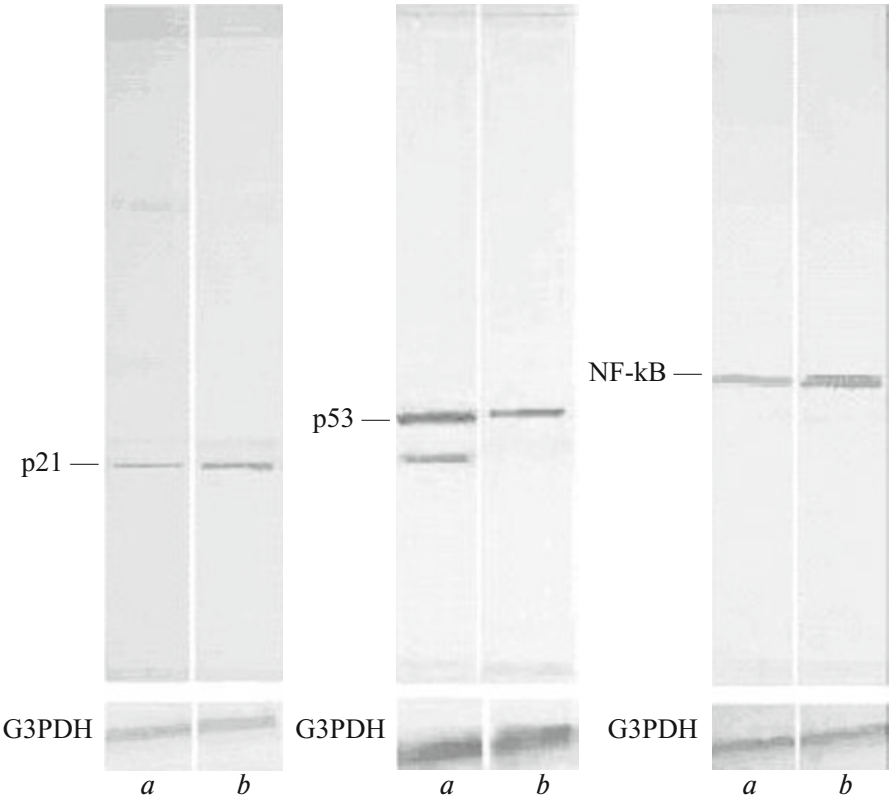

Fig. 2. Levels of p21, nonphosphorylated p53, and NF-kB RelA (p65) in blood lymphocytes. *a*) intact culture; *b*) cells incubated in medium with 0.05 ng/ml rTNF-α.

cytes cultured with rTNF-α in comparison with intact cells (Table 1; Fig. 2) indicates more intense formation of active p53. This can be caused by TNF receptor I-mediated stimulation of synthesis of ceramide and reactive oxygen forms promoting phosphorylation of p53 [2,4,8,12].

Realization of cell death program depends on the result of functioning of one more transcription factor, NF-kB [1]. Our experiments showed that the effects of rTNF-α on blood lymphocytes were associated with elevation of the content of NF-kB free subunit RelA (Table 1; Fig. 2), mediating the formation of many antiapoptotic proteins from the apoptosis inhibitor family (IAP) and of Bcl-2 [5]. The possibility of TNF-α-mediated stimulation of NF-kB via assembly of the specific signal complex was previously shown in HT1080 and 293T cells [9].

Our data indicate that the proapoptotic effect of p53 predominates over the NF-kB effect. This can be explained by mutual intranuclear competition of simultaneously stimulated p53 and NF-kB for binding to P300/CBP common coactivator complex providing interactions between the transcription factors and DNA [13]. Other intracellular events caused by successive (but not parallel) activation of p53 and NF-kB and leading to apoptosis cannot also be excluded. On the one hand, phosphorylated p53 stimulates (through MEK1 kinase) accumulation of NF-kB, which can later sensitize the cells to various apoptogenic factors [7,14], on the other hand, NF-kB-mediated activation of p53 leading to transcription of Bax and Bcl-xS proapoptotic protein genes was experimentally demonstrated not once [7].

Hence, our studies demonstrated a dose-dependent effects of TNF-α on cell death. Study of the mechanisms of the detected changes in the levels of p53, NF-kB, and p21 under conditions of apoptosis-inducing TNF-α effects will promote the development of molecular technologies of selective regulation of programmed death of immunocompetent cells.

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