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Granulocyte Differentiation Inducer, Hexapeptide HLDF-6, Decreases Cytotoxic Effect of Tumor Necrosis Factor on HL-60 Cell Line

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Abstract—The effect of hexapeptide HLDF-6, the granulocytic differentiation inducer, on the tumor necrosis factor α (TNF-α)-induced differentiation and apoptosis of human promyelocytic leukemia HL-60 cells has been investigated. Costimulation of HL-60 cells with HLDF-6 and TNF-α enhanced granulocyte differentiation, whereas the level of monocyte differentiation remained unchanged; however, the cytotoxic action of TNF-α on these cells decreased. The protective effect of HLDF-6 peptide did not depend on activation of NF-κB (nuclear transcription factor). Since HLDF-6 peptide decreases the number of cells entering apoptosis caused by C_2-ceramide, a mediator of TNF-induced apoptosis, and also reduces TNF-α-mediated activation of caspase-3, we have proposed the hypothesis that HLDF-6 increases resistance of HL-60 cells to the TNF-α cytotoxic effect due to inhibition of some stages of mitochondria-dependent apoptotic signaling.

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Key words: cell differentiation, apoptosis, peptide HLDF-6, TNF-α, HL-60 cell line, transcription factor NF-κB, ceramide

Apoptosis is a vital physiological process required for development, maintenance of tissue homeostasis, embryogenesis, and functioning of various differentiated cells [1-3]. Susceptibility of myeloid cell to various cytotoxic agents depends on their differentiation degree [4-7]. However, the precise relationship between cell differentiation and apoptosis remains unclear because mechanisms controlling these processes employ similar metabolic pathways [7]. So, elucidation of mechanisms responsible for regulation of apoptosis in tumor (undifferentiated) and normal (differentiated) cells is one of the key problems during chemotherapy of patients with various forms of leukemia. It is known that during differentiation myeloid cells acquire resistance to effects of various apoptosis inducers. For example, treatment of human promyelocytic leukemia cells (HL-60) with such differentiation inducers as phorbol esters, *all-trans*-retinoic acid, vitamin D_3, or dimethyl sulfoxide [8-15] caused their resistance to various apoptotic stimuli, including cytotoxic effect of tumor necrosis factor α (TNF-α) [16].

Our earlier work showed that hexapeptide HLDF-6, a fragment of an endogenous differentiation factor derived from HL-60 cells, human leukemia differentiation factor (HLDF), reproduced the whole differentiation activity of the full-size factor. Mechanism underlying the effect of HLDF-6 on HL-60 cells lacking their own plasma membrane receptor to this regulator involves peptide interaction with a lipid component of cell membranes, which alters membrane fluidity [17]. Studies revealed that besides differentiation activity, this hexa-

Abbreviations: BSA) bovine serum albumin; dNTP) 2′-deoxyribonucleoside triphosphate; DTT) dithiothreitol; FADD) Fas-associated death domain protein; FITC) fluorescein-5-isothiocyanate; GAPDH) glyceraldehyde-3-phosphate dehydrogenase; G-CSF) granulocyte colony-stimulating factor; G-CSFR) granulocyte colony-stimulating factor receptor; GM-CSF) granulocyte monocyte colony-stimulating factor; HLDF) human leukemia differentiation factor; IKK) I-κB kinases; I-κB) inhibitor of NF-κB; JNK) c-Jun N-terminal kinase; LPS) lipopolysaccharide; M-MLV) Moloney murine leukemia virus; NBT) nitroblue tetrazolium; NF-κB) nuclear factor κB; PE) phycoerythrin; PBS) phosphate-buffered saline; RIP) receptor-interacting protein; SAPKs) stress-activated protein kinases; SOD) superoxide dismutase; TNF-α) tumor necrosis factor α; TNF-Rs) tumor necrosis factor receptors; TRADD) TNF-R1-associated death domain protein; TRAF) TNF receptor-associated factor.

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peptide also exhibits protective properties. HLDF-6 protects Purkinje cells of rat cerebellar vermis *in vivo* and granular neurons of rat cerebellum *in vitro* under conditions of chemically induced hypoxia [18]. The hexapeptide also increased resistance of HL-60 cells and mouse embryos to cold shock and ionizing radiation [19]. HLDF-6 demonstrated neuroprotective effect in experimental models of Alzheimer's disease *in vivo* and *in vitro* [20]. Certain experimental evidence exists that TNF potentiates many of these processes [21].

TNF-α, a member of TNF-ligand family, is a multifunctional cytokine secreted by many cell types. TNF-α is a physiological inducer of such processes as apoptotic or necrotic cell death, cell differentiation, and hemopoiesis; it is also involved in regulation and realization of immune and inflammatory reactions [22]. Therapeutic use of TNF-α as an antitumor agent is complicated by its high general toxicity to normal cells, which is attributed to the wide spectrum of biological activity of this cytokine. Diverse biological effects of TNF-α are mediated by two high-affinity receptors, TNF-R1 and TNF-R2, which belong to a large family of TNF-receptors (TNF-Rs) [23]. In most cells, TNF-R1 plays a crucial role in TNF-α signal transduction; TNF-R2 functions in the lymphoid system [22]. The interaction of TNF-α with TNF-R1 and TNF-R2 causes three major effects underlying subsequent pathways for realization of various cell responses: a) activation of transcription factor NF-κB; b) activation of stress-activated protein kinases (SARKs/JNK); c) induction of apoptosis [22]. Initiation of apoptosis by TNF-α involves two pathways: direct receptor and mitochondrial.

The purpose of this study was the investigation of the influence of HLDF-6 peptide on processes of apoptosis and differentiation caused by TNF-α in human promyelocytic HL-60 cells, which is one of the stages in studying the molecular mechanism of action of this peptide.

MATERIALS AND METHODS

Reagents. The following reagents were used in this study: RPMI 1640 medium, fetal calf serum, nitroblue tetrazolium (NBT) (Sigma, Germany); a mixture of random hexanucleotide primers, kit for purification of PCR products "Wizard PCR Preps DNA Purification System", 2′-deoxyribonucleotide triphosphates (dNTPs), RNase inhibitor RNasin, Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, USA); RNase inhibitor RNaseZAP (Invitrogen, USA); "PCR MIMIC^{TM} Construction Kit" (Clontech, USA); "Annexin V-FITC Apoptosis Detection Kit" (PharMingen, USA); Coomassie R-250, Coomassie G-250, agarose (Bio-Rad, USA); "ECL+Plus Detection System" kit (Amersham Biosciences, USA); *Taq*-polymerase (Laboratory of Biotechnology, Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia). All other reagents of "specially pure grade" or "chemically pure grade", used for preparation of buffer solutions, were produced by local suppliers.

Human recombinant TNF-α was kindly provided by L. N. Shingarova, and C_2-ceramide was a generous gift of Yu. G. Molotkovsky (both from the Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia).

HL-60 cell line was kindly given by R. G. Vasilov (Institute of Biotechnology, Russia).

Cultivation of HL-60 cells was carried out in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in atmosphere of 5% CO_2.

Differentiation activity of HL-60 cells was assayed by the nitroblue tetrazolium dye reduction test (NBT test) using a standard method [24]. HL-60 cells ($5 \cdot 10^6$) were incubated with TNF-α (1 μg/ml), HLDF-6 (10 μM), or TNF-α (1 μg/ml) + HLDF-6 (10 μM) for 72 h. After the incubation, NBT (1 μg/ml) was added to cell pellet and the mixture was incubated for 25 min at 37°C and 15 min at room temperature. After the incubation, cells were uniformly distributed onto a glass slide (smear) and fixed in methanol for several minutes. Calculation of cells containing dark blue cytoplasmic granules of formazan (reduced NBT) was carried out using a LOMO microscope (Russia). In each sample, at least 300 cells were counted. Cells containing at least 10 formazan granules were considered as the differentiated ones. Cells incubated in phosphate-buffered saline (PBS) were used as control. There were three independent experiments (each in triplicate).

Total RNA was isolated from $2 \cdot 10^6$ cells treated with the inducers for 1, 4, 24, 48, and 72 h using RNA isolation kit "SV Total RNA Isolation System" (Promega) and the supplier's isolation protocol. Samples were also treated with DNase to remove possible contaminations of RNAs with genomic DNA.

Preparation of cDNA. For reverse transcription, we used 1 μg RNA from each sample. Reverse transcription was carried out in the system (final volume 20 μl) containing 10 mM dNTP, 75 mM KCl, 5 mM dithiothreitol (DTT), 50 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 0.002% bovine serum albumin (BSA), 100 ng of random hexanucleotide primers, 20 U of RNasin, 200 U M-MLV reverse transcriptase (all reagents from Promega). The reaction was carried out at 37°C for 1.5 h, and the enzyme was inactivated by incubation at 95°C for 15 min. The volume of the reaction mixture was adjusted up to 500 μl with sterile Milli-Q water. Quality of isolated RNA and effectiveness of reverse transcription were evaluated using PCR and DGAP and RGAP as primers to cDNA template of glyceraldehyde-3-phosphate dehydrogenases (GAPDH) (Table 1).

Semiquantitative PCR. For PCR reaction, competitive templates (competitors) were synthesized. They did

Table 1. Structure of oligonucleotides used for analysis of expression of TNF-α, CD114, and CD11b

Structure of oligonucleotides (5′→3′)	Size of amplified DNA products, bp	
	tested template	competitive template
GAPDH		
DGAP: tgcaccaccaactgcttagc		
RGAP: cattgtcataccaggaaatgagc	495	
DGAPC: tgcaccaccaactgcttagctgttatacagggagatgaaa		
RGAPC: cattgtcataccaggaaatgagcatttgattctggaccatggc		367
TNF-α		
DTNF: gtttccagacttccttgagacac		
RTNF: taggccgattacagacacaac	550	
DTNFC: gtttccagacttccttgagacaccgcaagtgaaatctcctccg		
RTNFC: taggccgattacagacacaacatttgattctggaccatggc		375
CD114 (G-CSFR)		
DCD114: cctggagctgagaactac		
RCD114: tcccggctgagttatagg	274	
CD11b		
DCD11b: atcaatatcaggtcagcaacc		
RCD11b: gaggtcttgatgtaccagtc	321	

not share homology with templates used but had common sequences for primer annealing. The non-homologous competitive templates (competitors) were obtained using "PCR MIMIC™ Construction Kit" and the supplier's protocol. Amplification products from the competitive templates differed in length from the studied templates. We used the average difference of 200 bp. This was necessary for identification of PCR products by electrophoresis. For cDNA calculation in each sample, we synthesized GAPDH competitor of 367 bp using DGAPC and RGAPC primers (Table 1). Similarly, a competitor of 550 bp was synthesized using DTNFC and RTNFC primers (Table 1) for evaluation of TNF-α expression. The resulting competitive templates were then purified using "Wizard PCR Preps DNA Purification System" and the supplier's protocol. Concentrations of the competitors obtained were measured spectrophotometrically at 260 nm. Total cDNA amount for each experiment was normalized by GAPDH cDNA; its concentration in all samples was analyzed by means of competitive PCR.

Semiquantitative PCR was carried out using the calculated amount of total cDNA containing the same amount of GAPDH cDNA and TNF-α competitor in each sample. The reaction mixture (of 25 μl) for competitive PCR contained 67 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.25 mM dNTP, 0.01% Tween-20, 5 pM gene-specific primers, 0.1 U Taq DNA polymerase. The reaction was carried out using PTC-200 amplifier (MJ Research, USA) and the following temperature regime: 94°C for 90 sec, 35 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec followed by a final extension of 5 min at 72°C. After electrophoresis in 2% agarose gel and staining with ethidium bromide followed by UV visualization, there were two PCR-products for each reaction. Electrophoretic images were treated using the Scion Image for Windows program v.4.0.2. Data were represented as a ratio of the amount of PCR-product obtained on a tested template to the amount of PCR product obtained on the competitive template; this ratio characterized relative amount of investigated cDNA in a particular sample. There were at least three independent experiments in pentaplicates.

Reverse transcription PCR (RT-PCR). For analysis of expression of CD11b and CD114, cells were stimulated by the inducers for 24 h. Isolation of total RNA and cDNA synthesis were carried out as above. Initially for evaluation of expression of these antigens, we normalized the amount of total cDNA by means of GAPDH cDNA; its amount was analyzed in all samples by competitive PCR as above. The reaction mixture (of 25 μl) for com-

petitive PCR contained 67 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.01% Tween-20, 5 pM gene-specific primers (Table 1), 0.1 U Taq DNA polymerase. The reaction was carried out using the PTC-200 amplifier and the following temperature regime: 94°C for 90 sec, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec followed by a final extension of 5 min at 72°C. There were at least three independent experiments in pentaplicates.

Level of cell death was evaluated by Trypan blue staining. HL-60 cells ($5 \cdot 10^6$) were incubated with TNF-α (1 μg/ml), HLDF-6 (10 μM), or TNF-α (1 μg/ml) + HLDF-6 (10 μM) for 4, 24, and 72 h. After the incubation, aliquots of cell suspension were stained with Trypan blue. Cell death evaluated as difference in number of stained cells in experimental and control samples was expressed in percent. There were at least three independent experiments in triplicates.

Apoptosis in cells was monitored by cytofluorimetry using "Annexin V-FITC Apoptosis Detection Kit" and a FC 500 flow cytofluorimeter (Beckman, USA). Cells were incubated for 24 h in the presence of the investigated compounds, washed with cold PBS, and then stained with FITC-labeled annexin V and propidium iodide following the supplier's recommendations. Cells incubated in PBS were used as control. In each experiment, at least 10,000 events were analyzed. There were at least three independent experiments in triplicates.

Activity of cell caspases-8 and -3 were evaluated by the amount of *p*-nitroaniline (pNA) formed during hydrolysis of modified substrates of caspase-3 (Ac-Asp-Glu-Val-Asp-pNA) and caspase-8 (Ac-Ile-Glu-Thr-Asp-pNA) using a "Caspase-3,-8 Assay Kit, Colorimetric" (Sigma, USA). HL-60 cells ($5 \cdot 10^6$) were incubated with TNF-α (1 μg/ml), HLDF-6 (10 μM), or TNF-α (1 μg/ml) + HLDF-6 (10 μM) for 24 h and then lysed with buffer containing 50 mM Hepes (pH 7.4), 1 mM DTT, 0.1 mM EDTA, 0.1% CHAPS, and 0.1% Triton X-100. According to the supplier's instructions, caspase substrates were added to the lysate of cells (~$400 \cdot 10^3$ cells) in the final concentration of 200 μM. The final volume of the reaction mixture was 100 μl. The incubation with substrates was carried out at 37°C for 2 h. Absorbance of pNA was read at 405 nm using a Multiscan MCC/340 (Labsystems, Finland). Results were expressed in percent. The amount of pNA formed in a control sample was defined as 100%. There were at least three experiments.

Immunoblot analysis. Cells ($5 \cdot 10^6$) incubated with the studied compounds for 30 min, 1 h, and 4 h were pelleted, washed with cold PBS, and resuspended in the lysing buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.2 M DTT, 0.2% Bromophenol Blue). An aliquot of cell lysate (~$200 \cdot 10^3$ cell) preincubated at 100°C for 7 min was subjected to electrophoresis in 10% SDS-polyacrylamide gel followed by subsequent transfer to Immobilon-P membrane (Millipore, USA). The membranes incubated for 1 h in PBS containing 5% defatted dry milk were then subjected to overnight incubation with primary antibodies (1 : 1000) at 4°C. After washing for 1 h, the membranes were treated with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology, USA) (1 : 3000). The binding of antibodies was evaluated by chemiluminescence on a radioautographic film using the "ECL+Plus detection system" kit. Antibodies to GAPDH were used for evaluation of total protein content. The following primary polyclonal antibodies were used: antibodies against GAPDH (Santa Cruz Biotechnology), antibodies against kinases IKKα and IKKβ and to phosphorylated forms of these kinases (p-IKKα and p-IKKβ), and antibodies against RelA/p65 subunit and its phosphorylated form (p-RelA/p65) (Cell Signaling Technology, USA).

Cell cycle was analyzed using the fluorescent dye propidium iodide and the method of flow cytofluorimetry. The measurements were carried out on an FC 500 flow cytofluorimeter. Cells ($9 \cdot 10^6$) incubated with the inducers for 48 h were sedimented, washed twice with cold PBS, and fixed in 70% ethanol for 30 min at 4°C. After fixation cells were washed again in cold PBS and resuspended in 500 μl of PBS containing 1 μg/ml of propidium iodide and 10 μg/ml RNase A (Sigma, Germany). The samples were incubated at 37°C for 1 h and distribution was analyzed using the FC 500 flow cytofluorimeter. In each experiment, at least 10,000 events were analyzed. There were at least three independent experiments.

Expression of the differentiation markers CD14 and CD16 was analyzed by the cytofluorimetry using the FC 500 flow cytofluorimeter. Cells ($9 \cdot 10^6$) incubated with the inducers for 72 h were sedimented, washed twice with cold PBS, and incubated with phycoerythrin (PE)-labeled antibodies against CD14 and CD16 (Beckman Counter Immunotech, France) at 25°C for 30 min. Nonspecific binding was monitored using PE-labeled IgG (Beckman Counter Immunotech). In each experiment, at least 10,000 events were analyzed. There were at least three independent experiments in triplicates.

Statistical analysis. The experimental results were analyzed by Student's paired *t*-test.

RESULTS

Hexapeptide HLDF-6 induces granulocytic type of differentiation, whereas TNF-α induces monocytic type of differentiation of HL-60 cells. Employment of the standard NBT test reflecting metabolic activity of cells [25] revealed that hexapeptide HLDF-6 and TNF-α induce differentiation of HL-60. Incubation of HL-60 cells with these inducers for 72 h caused twofold increase in the number of cells reducing NBT (Fig. 1). Simultaneous treatment of HL-60 cells with TNF-α and HLDF-6 pep-

tide caused statistically significant (but not additive) increase in NBT-positive cells; the latter clearly indicates an increase in the differentiation process. Using cytofluorimetry, we analyzed the type of cell differentiation induced by these agents. Analysis of CD14 expression (specific marker of monocytic differentiation) by means of PE-labeled antibodies against CD14 revealed that incubation of HL-60 cells with TNF-α for 72 h significantly increased number of CD14 positive cells (Table 2). On treatment of cells with TNF-α and HLDF-6, the number of CD14(+) cells remained unchanged compared with the independent effect of TNF-α. HLDF-6 peptide alone did not influence expression of this differentiation marker. Using a similar approach, we analyzed the effects of these inducers on the expression of CD16, the marker of neutrophil/granulocyte type of differentiation. Treatment with TNF-α caused a 1.5-fold increase in number of CD16(+) cells, whereas HLDF-6 peptide did not influence number of cells with CD16(+) phenotype. Co-treatment of cells with these inducers caused the same effect on number of CD16(+) cells as incubation with TNF-α only. Expression of two other superficial antigens, CD11b and CD114 (G-CSF receptor), was analyzed by RT-PCR. cDNA was synthesized on total RNA isolated from cells treated with TNF-α and HLDF-6 peptide for 24 h. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal standard.

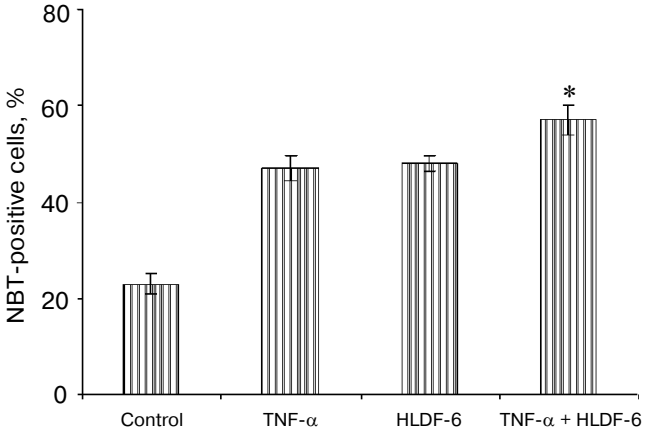

Fig. 1. Effect of HLDF-6 on TNF-induced HL-60 cell differentiation. Results of the NBT test obtained after treatment of cells with TNF-α (1 μg/ml), HLDF-6 (10 μM), or TNF-α (1 μg/ml) + HLDF-6 (10 μM) for 72 h are expressed as percent. Cells incubated with PBS were used as control. Data represent mean ± SD of three independent experiments. Asterisk shows statistically significant (*$p < 0.05$) increase in NBT-positive cells compared with cells treated with TNF-α or HLDF-6 only.

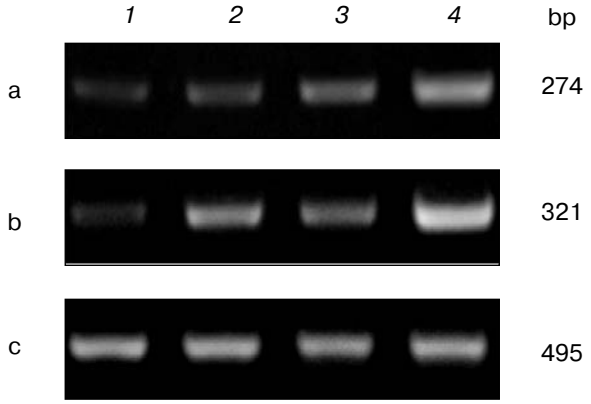

Fig. 2. Effect of TNF-α and HLDF-6 on expression of differentiation markers CD114 and CD11b. Results of PCR on cDNA template obtained from HL-60 cells treated with the inducers for 24 h: *1*) PBS; *2*) TNF-α (1 μg/ml); *3*) HLDF-6 (10 μM); *4*) TNF-α (1 μg/ml) + HLDF-6 (10 μM). a-c) PCR products obtained using cDNA templates of antigens CD114, CD11b, and GAPDH, respectively.

Table 2. Effect of HLDF-6 and TNF-α on expression of differentiation markers CD14 and CD16

Inducer	Number of cells, %*	
	CD14(+)	CD16(+)
Control	2.8 ± 0.4	22.0 ± 0.6
TNF-α	54.6 ± 2.7	32.7 ± 1.8
HLDF-6	3.0 ± 0.6	21.1 ± 0.4
TNF-α + HLDF-6	51.1 ± 2.8	34.2 ± 1.4

* Number of CD14(+) and CD16(+) cells was determined cytofluorimetrically after staining with PE-labeled antibodies against CD14 and CD16. HL-60 cells were incubated with TNF-α (1 μg/ml), HLDF-6 (10 μM), or TNF-α (1 μg/ml) + HLDF-6 (10 μM) for 72 h. Data represent mean ± SD of three independent experiments.

As suggested, incubation of HL-60 cells with TNF-α or HLDF-6 for 24 h increased the expression of CD11b, the marker of cell differentiation [16, 26]; costimulation of these cells with TNF-α and the peptide caused more pronounced increase in the amount of its transcript (Fig. 2). These results are consistent with the fact that HLDF-6 peptide caused granulocytic differentiation: it was demonstrated earlier that neutrophil/granulocyte differentiation induced in HL-60 cells by such inducers as all-*trans*-retinoic acid and dimethyl sulfoxide (DMSO) was accompanied by increased expression of CD11b but not CD16 [27]. Results of the study of TNF-α and HLDF-6 effects on the expression of CD114 in HL-60 cells are also consistent with granulocytic type of differentiation induced by this hexapeptide. The level of CD114 mRNA in cells treated with TNF-α for 24 h was comparable to the control level; the latter is consistent with numerous data on predominance of monocytic type of differentia-

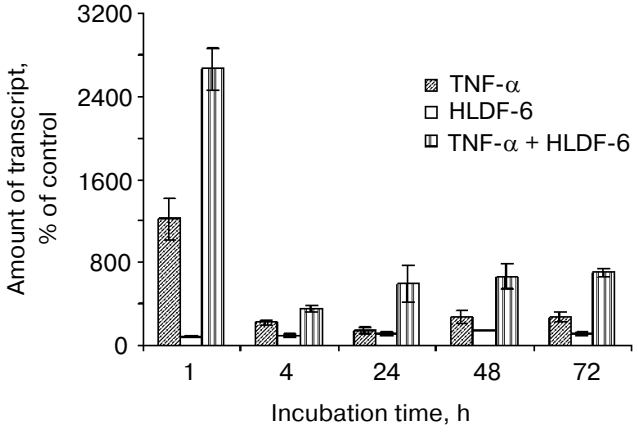

Fig. 3. Competitive PCR analysis of the level of endogenous TNF-α expression induced by cell treatment with TNF-α and HLDF-6. cDNA was isolated from cells treated with TNF-α (1 μg/ml), HLDF-6 (10 μM), or TNF-α (1 μg/ml) + HLDF-6 (10 μM) during the indicated time intervals. Cells incubated with PBS were used as control. The abscissa shows relative amount of TNF-α transcript expressed as % of control.

tion induced by this cytokine [28]. Peptide HLDF-6, the inducer of granulocytic differentiation, caused the increase in CD114 transcript in HL-60 cells. However, simultaneous treatment of cells with TNF-α and HLDF-6 peptide caused sharp increase in CD114 antigen transcript (Fig. 2).

HLDF-6 increases TNF-mediated expression of endogenous TNF-α. Since TNF-α is considered as an endogenous autocrine differentiation factor [29], we investigated the effect of exogenous TNF-α on the expression of endogenous TNF-α. Qualitative evaluation of TNF-α expression by RT-PCR did not give clear results. So we analyzed expression of this gene using semiquantitative competitive PCR (QC-RT-PCR), as the most sensitive and convenient method for testing of a large number of samples. The following result was obtained: the level of endogenous TNF-α after treatment of cells with HLDF-6 did not vary significantly (Fig. 3). Treatment of HL-60 cells with exogenous TNF-α for 1 h caused a 10-fold increase in TNF-α transcript; prolonged incubation for 4 h resulted in sharp decrease in TNF-α transcript, which, however, exceeded control value up to 72 h incubation. Simultaneous treatment of cells with TNF-α and HLDF-6 for 1 h caused much higher (~26-fold) increase in TNF-α transcript level, which also decreased after incubation for 4 h but was significantly higher than the corresponding level of TNF-α transcript in HL-60 cells treated with exogenous TNF-α for 4 h. Subsequent incubation of cells with both inducers resulted in the increase in TNF-α transcript level which was 6 times higher than in control cell (24-h incubation) and remained at this level up to incubation for 72 h (Fig. 3). Consequently, augmentation of TNF-induced differentiation of HL-60 cells caused by HLDF-6 peptide was accompanied by prolonged secretion of endogenous TNF-α. This also suggests increased cell differentiation.

HLDF-6 and TNF-α arrest cell division. The effect of these compounds on the cell cycle was evaluated by content of duplicated and non-duplicated DNA using flow cytometry with the fluorescent dye propidium iodide.

Treatment of cells with HLDF-6 hexapeptide for 48 h resulted in accumulation of cells in G_0/G_1 phase and reduction of cells at S-phase (Fig. 4). Number of cells at G_2/M phase remained unchanged. Incubation of cells in the presence of TNF-α was also accompanied by arrest of cells in G_0/G_1 phase, but their distribution pattern was different. Treatment with TNF-α was accompanied by reduction in percent of cells in G_2/M phase, but increased the region reflecting number of hypo-diploid (apoptotic) cells. Costimulation of cells with both TNF-α and HLDF-6 resulted in higher accumulation of cells in G_0/G_1 phase compared with independent effects of the inducers; number of cells in G_2/M phase increased compared to that in case when TNF-α alone was used. The percent of cells in S-phase was somewhat higher than in the case of their treatment with TNF-α only, but lower than in the case of cell treatment with HLDF-6 peptide. The number of hypo-diploid cells did not differ from the control level.

Since costimulation of cells is accompanied by reduction in number of apoptotic cells compared with independent action of TNF-α, we have suggested that simultaneous treatment of HL-60 cells with TNF-α and HLDF-6 leads to appearance of cell resistance to cytotoxic effect of TNF-α.

HLDF-6 attenuates cytotoxic effect of TNF-α. The protective effect of HLDF-6 peptide has been investigat-

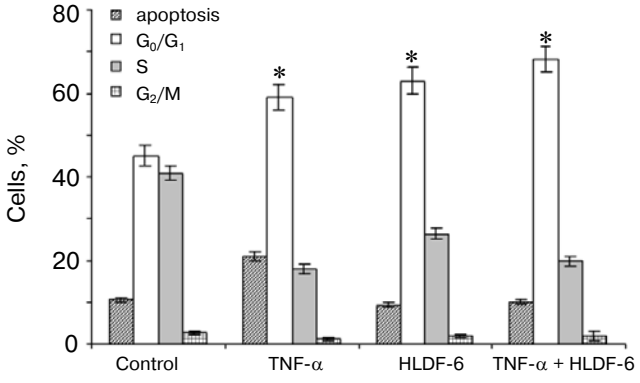

Fig. 4. Effect of TNF-α and HLDF-6 on cell cycle. Cells stained with propidium iodide were analyzed by cytofluorimetry. HL-60 cells were treated with TNF-α (1 μg/ml), HLDF-6 (10 μM), or with combination of TNF-α (1 μg/ml) and HLDF-6 (10 μM) for 48 h. Data represent mean ± SD of three independent experiments. Asterisk shows statistically significant ($p < 0.05$) increase in percent of cells at G_0 compared with control.

ed using two independent methods: Trypan blue staining and flow cytofluorimetry (double staining with FITC labeled annexin V and propidium iodide).

In the first set of experiments cells were treated with these inducers for 4, 24, and 72 h. Treatment of cells with TNF-α was accompanied by significant increase in dead cells during 24 and 72 h (Fig. 5). HLDF-6 did not influence the process of cell death (Fig. 5). However, in the presence of HLDF-6 cell resistance to cytotoxic effect of TNF-α increased.

These data were further confirmed by subsequent cytofluorimetric analysis of HL-60 cells treated for 24 h. It was shown that TNF-α increases number of annexin-V(+)- and PI(+)-cells (i.e., cells with induced apoptosis), whereas HLDF-6 peptide is not an apoptosis inducer (Table 3). Simultaneous cell treatment with TNF-α and HLDF-6 peptide decreased the number of apoptotic cells almost to the control level. Consequently, the differentiating effect of HLDF-6 peptide is also accompanied by increased resistance of HL-60 to the cytotoxic effect of TNF-α.

The mechanism of protective effect of HLDF-6 hexapeptide is not related to activation of transcription factor NF-κB. In many cells TNF-α is known to cause not only induction of the apoptotic cascade, but also activation of transcription factor NF-κB, which controls expression of many genes involved in regulation of proliferation, inflammation, and apoptosis [22].

The cascade of reactions resulting in NF-κB induction includes phosphorylation of regulatory kinase complex, which in turn phosphorylates IκB protein, an inhibitor of transcription factor NF-κB. This chain of events results in release and translocation into the nucleus of active NF-κB subunits RelA/p65 and RelB/p68 [22]. The transcription activity of NF-κB correlates with phosphorylation of Ser536 residue of RelA/p65 [30]. We evaluated NF-κB activation by the increased phosphorylation level of regulatory kinase complex (IKK) and transcriptionally active subunit, RelA/p65, by immunoblot analysis. Treatment of cells with TNF-α for 30 min and 1 h caused significant increase in phosphorylation level of IKK kinase complex; during subsequent incubation the phosphorylation level decreased to the control level (Fig. 6). The presence of HLDF-6 peptide did not influence TNF-α-induced phosphorylation of IKK kinase complex. The phosphorylation level of IKK kinase complex of HL-60 cells treated with HLDF-6 peptide was comparable to the control level. Similar results have been obtained during evaluation of p65 subunit phosphorylation degree. It should be noted that expression of proteins of the kinase complex and p65 remained unchanged over the studied incubation period.

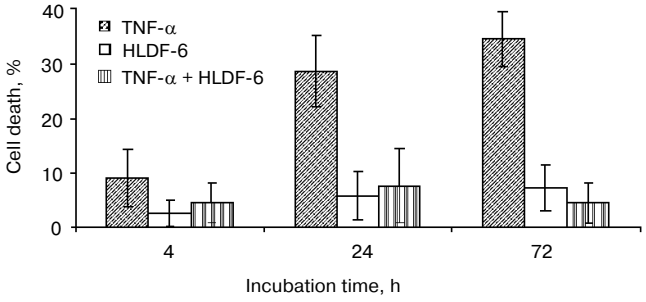

Fig. 5. Effect of HLDF-6 on TNF-induced death of HL-60 cells. The number of dead cells was evaluated by staining with Trypan Blue after incubation of cells with TNF-α (1 μg/ml), HLDF-6 (10 μM), or with combination of TNF-α (1 μg/ml) and HLDF-6 (10 μM) during the indicated time intervals. In experimental samples, the number of control cells was subtracted from total number of dead cells. Data expressed in percent represent mean ± SD of three independent experiments.

Table 3. Effect of TNF-α and HLDF-6 on number of apoptotic cells

Inducer	Number of apoptotic cells, %*
Control	7.6 ± 1.1
TNF-α	21.5 ± 3.3
HLDF-6	9.3 ± 1.3
TNF-α + HLDF-6	10.4 ± 1.6

* The number of apoptotic cells was determined by cytofluorimetry after double staining of cells with FITC-labeled annexin V and propidium iodide. HL-60 cells were incubated with TNF-α (1 μg/ml), HLDF-6 (10 μM), or with combination of TNF-α (1 μg/ml) and HLDF-6 (10 μM) for 24 h. Data represent mean ± SD of three independent experiments.

HLDF-6 decreases TNF-induced activation of caspase-3 but increases activation of caspase-8. We also investigated the effects of these agents on activation of initiation caspase-8 and key effector caspase-3 using the colorimetric method. Treatment of HL-60 cells with TNF-α for 24 h caused significant activation increase of both caspase-3 and caspase-8 (Fig. 7a). HLDF-6 alone did not influence activity of these enzymes, but attenuated TNF-induced activation of caspase-3 and significantly potentiated TNF-induced activation of caspase-8 (Fig. 7b).

Hexapeptide HLDF-6 abolishes ceramide-induced apoptosis. Using cytofluorimetry (double staining of cells with FITC-labeled annexin V and propidium iodide), we investigated the effect of HLDF-6 on cell death induced by C_2-ceramide, a well-known inducer of apoptosis. Treatment of cells with 1 μM C_2-ceramide for 24 h was accompanied by appearance of a significant number of cells entering apoptosis. Addition of 10 μM HLDF-6 to the incubation medium reduced the number of apoptotic cells to the control level (Table 4).

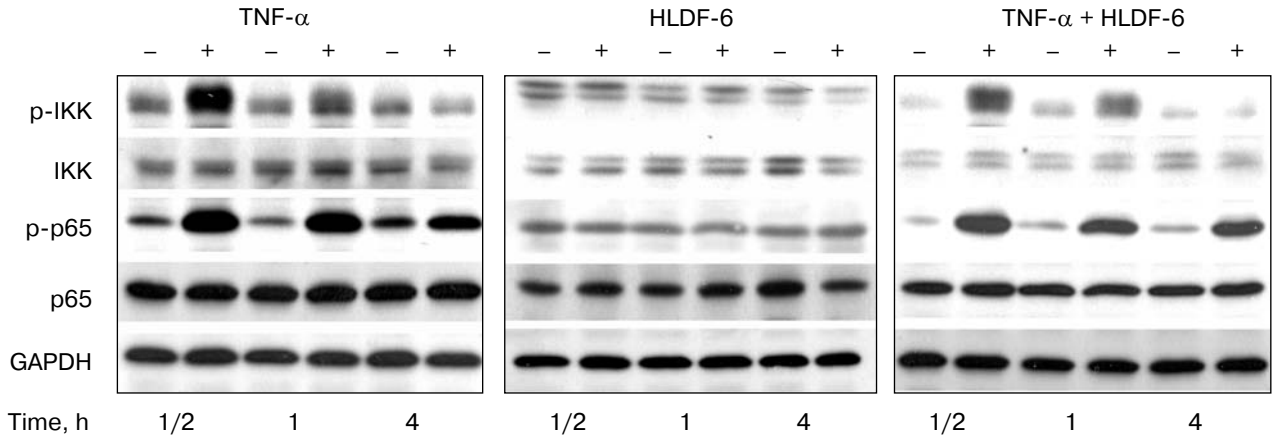

Fig. 6. Immunoblot analysis of IKK kinase complex and RelA/p65 and their phosphorylated forms, p-IKK and p-p65, respectively. Their altered phosphorylation level in HL-60 cells shown here was evaluated using antibodies to IKK kinase complex, RelA/p65 subunit, and corresponding phosphorylated forms. Designations: –, cells incubated with PBS; +, cells incubated with TNF-α (1 μg/ml), HLDF-6 (10 μM), or with combination of TNF-α (1 μg/ml) and HLDF-6 (10 μM) for 30 min and 1 or 4 h.

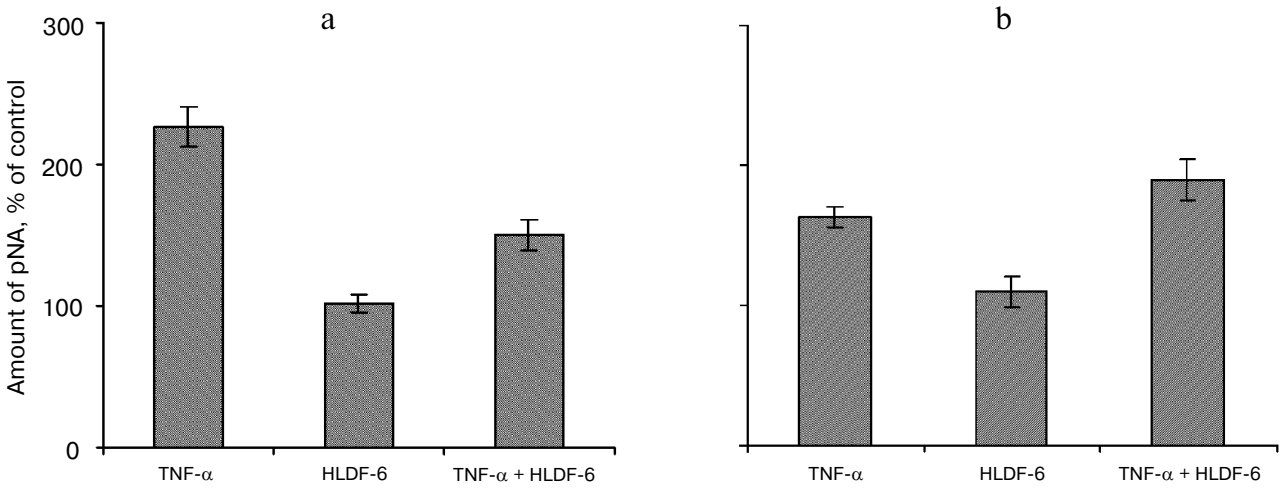

Fig. 7. Effect on HLDF-6 on TNF-α-induced activation of caspase-3 (a) and caspase-8 (b). Caspase activity was evaluated colorimetrically by the appearance of *p*-nitroaniline (pNA) formed during hydrolysis of modified substrates of caspase-3 (Ac-DEVD-pNA) and caspase-8 (Ac-IETD-pNA) after incubation with cell lysates. HL-60 cells were incubated with TNF-α (1 μg/ml), HLDF-6 (10 μM), or TNF-α (1 μg/ml) + HLDF-6 (10 μM) for 24 h. The amount of pNA formed in the control sample was defined as 100%. Data represent mean ± SD of three independent experiments.

DISCUSSION

Clinical use of TNF-α as an antitumor agent is limited by its potent toxic effect. Current strategy for the development of therapeutic methods of its use involves the combination of this cytokine with various differentiation inducers of chemical (all-*trans*-retinoic acid, dimethyl sulfoxide, phorbol esters) or biological (growth factors GM-CSF and G-CSF, interferon) origin; this promotes the differentiation effect of TNF-α on tumor cells and decrease of its toxic effect on the organism [31-34]. Biologically active peptide HLDF-6, being a granulocytic differentiation inducer of myeloid cells [17], can become one such agents.

We investigated the influence of HLDF-6 peptide on TNF-mediated effects on human promyelocytic leukemia (HL-60) cells. Using the NBT test and analysis of CD11b expression, we demonstrated that combined treatment of these cells with TNF-α and HLDF-6 hexapeptide results in increase in cell differentiation (Figs. 1 and 2). Taking into consideration that HL-60 cells are pluripotent cells, which may undergo further differentia-

Table 4. Effect of C_2-ceramide and HLDF-6 on number of apoptotic cells

Inducer	Number of apoptotic cells, %*
Control	13.3 ± 1.4
C_2-Cer	33.7 ± 3.7
HLDF-6	13.6 ± 1.5
C_2-Cer + HLDF-6	14.8 ± 1.6

* The number of apoptotic cells was determined by cytofluorimetry after double staining of cells with FITC-labeled annexin V and propidium iodide. HL-60 cells were incubated with C_2-ceramide (1 μM), HLDF-6 (10 μM), or with combination of C_2-ceramide (1 μM) and HLDF-6 (10 μM) for 24 h. Data represent mean ± SD of three independent experiments.

tion along (TNF-α and vitamin D_3-induced) monocyte/macrophage or (all-*trans*-retinoic acid and DMSO-induced) neutrophil/granulocyte pathways, we analyzed phenotype of the differentiated cells [35, 36]. The use of CD14, the expression marker of monocyte differentiation, revealed that costimulation of HL-60 cells with TNF-α and HLDF-6 peptide insignificantly influenced number of C14-positive cells compared with the independent effect of TNF-α (Table 2). The same situation was also observed in the case of expression analysis of CD16, which is expressed by monocytes and neutrophils at the stage of terminal differentiation [36]. HL-60 cells differentiated along the granulocyte pathway differ from their mature analogs and do not necessarily express the same antigens. In some cases, even treatment of HL-60 with such potent granulocyte differentiation inducer as all-*trans*-retinoic acid was not accompanied by any detectable expression of CD16 on the cell surface [27]. Analysis of CD114 expression typical for early stage of differentiation along the granulocyte pathway [37] revealed that combined treatment of HL-60 cells with TNF-α and HLDF-6 peptide resulted in the increase in expression of this antigen (Fig. 2). Thus, combined treatment of the culture of HL-60 cells with TNF-α and HLDF-6 caused potentiation of HLDF-6-induced neutrophil/granulocyte differentiation.

Endogenous TNF-α is an autocrine differentiation factor and blockade of its biosynthesis during stem cell differentiation by the growth factor GM-CSF was accompanied by the increase of their proliferation [38]. Increased secretion of endogenous TNF-α is one of the characteristic signs of myeloid cell differentiation; it is related to increased level of expression of cell adhesion factors and increased production of free oxygen radicals and nitric oxide [29, 39]. Biosynthesis of endogenous TNF-α may also account for the development of myeloid cell resistance to stressful treatments (UV, lipopolysaccharide, exogenous TNF-α) [40-42]. Studies of the effect of the hexapeptide on regulation of expression of endogenous TNF-α by exogenous TNF-α revealed stepwise increase in TNF-α endogenous transcript in the process of this incubation. At the initial stage of incubation (1 h) with TNF-α, or TNF-α + HLDF-6 the amount of this transcript sharply increased and then sharply decreased after 4 h incubation. Costimulation caused more than 2-fold increase in transcript accumulation compared with independent effect of TNF-α. Treatment of cells for 24 h was accompanied by a second prolonged increase in secretion of endogenous TNF-α, typical for cells that had undergone the initial stage of differentiation. Incubation with only exogenous TNF-α for 48 h caused 2-fold increase in expression of endogenous TNF-α, whereas in combination with HLDF-6 already in 24 h there was more than 6-fold increase in the amount of TNF-α mRNA. Consequently, treatment of HL-60 cells with HLDF-6 involves TNF-mediated recover of secretory functions of cells typical for differentiated myeloid cells.

Myeloid cell differentiation is accompanied by changes in the cell cycle: cells are accumulated in G_0 phase (resting phase) and the number of cells in S-phase (phase of DNA synthesis) decreased [43]. Analysis of the cell cycle revealed that both HLDF-6 peptide and TNF-α caused preferential cell entry into G_0 phase accompanied by corresponding decrease in cells in S-phase (Fig. 4). Combined treatment of cells with TNF-α and HLDF-6 was accompanied by significant increase in the number of cells in G_0. Under these conditions, percent of cells in S-phase was somewhat higher than in the case of independent effect of TNF-α, but lower than in the case of cell treatment with HLDF-6 only. However, the number of hypo-diploid cell did not differ from the control value. Although HLDF-6 peptide increased the number of cells in G_2/M phase, this phenomenon was not related to the increase in proliferation, because we earlier demonstrated that HLDF-6 inhibited incorporation of [^{3}H]thymidine [17]. There is evidence that during differentiation accumulation of cells in G_2/M phase precedes transition into G_0/G_1-phase and number of cells in S-phase decreases [44]. Using our approach, we could not detect number of cells approaching G_2 and M phases separately, but it was shown that phorbol ester-induced differentiation of myeloid cells was accompanied by accumulation of cells in G_2-phase and blockade of transition into mitotic phase [45]. Cell arrest in G_2/M suggests activation of processes related to structural rearrangements of DNA and its repair preventing cell death [43]. Experiments revealed that costimulation of HL-60 cells with TNF-α and HLDF-6 for 24 h reduced percent of cells in the phase reflecting number of apoptotic (hypo-diploid) cells compared with the effect of TNF-α only. These data suggest that combined treatment of HL-60 cells with TNF-α and HLDF-6 caused resistance of these cells to the toxic effect of TNF-α. This hypothesis has been confirmed using two independent methods, staining with Trypan

blue and flow cytofluorimetry with double staining of cells with FITC-labeled annexin V and propidium iodide (Fig. 5 and Table 3).

In contrast to well-studied TNF-induced cell death, molecular mechanism underlying resistance of cells to the cytotoxic effect of this cytokine definitely requires detailed investigation. After TNF-α binding to its receptor, TNF-R1, subsequent signal transduction involves formation of two spatially independent molecular complexes [46]. Interaction of TNF-α with its receptor causes initial formation of "complex I" containing receptor itself and also adaptor proteins: RIP1, TRAF2, and TRADD. Such complex transforms signals leading to activation of transcription factor NF-κB and/or stress-activated protein kinases (SARKs/JNK). Subsequently, signal transduction involves dissociation of "complex I" and formation of "complex II" (DISC or "death complex"), which also includes adaptor protein FADD and procaspase-8. "Complex I" plays a regulatory role: its regulation of NF-κB controlling expression of antiapoptotic genes determines the possibility of "complex II"-induced apoptosis [46].

It would be reasonable to suggest that the effect of HLDF-6 peptide is realized via biosynthesis of receptors TNF-R1 and TNF-R2. However, analysis of expression of TNF-α receptors revealed that incubation of cells with the agents studied insignificantly influenced the level of receptor mRNA. (This was demonstrated using semi-quantitative PCR, data not shown.)

We investigated the effects of HLDF-6 and TNF-α on activation of transcription factor NF-κB, because good evidence exists that increase in HL-60 cell survival is associated with its increased activity [47]. The data suggest that the mechanism responsible for the development of HLDF-6-induced resistance of HL-60 cells to TNF-induced death does not involve the increase in NF-κB activity, and HLDF-6 does not influence TNF-mediated signal transduction by "complex I" (Fig. 6). For analysis of the effect of HLDF-6 on transduction of TNF-apoptotic signal by "complex II", we investigated the role of these inducers in activation of the initiator caspase-8 and also key effector caspase-3. Besides the direct receptor mediated pathway employing "complex II" and caspase-8, TNF-induced activation of effector caspase-3 also occurs via the mitochondrial apoptotic pathway. The latter involves mitochondrial membrane depolarization and release of cytochrome *c* forming the apoptosome with Apaf-1 and procaspase-9, leading to activation of caspase-9, which in its turn activates caspase-3 [48]. Taking into consideration that simultaneous action of HLDF-6 and TNF-α on HL-60 cells caused an increase in caspase-8 activity and the decrease in caspase-3 activity compared with the independent effect of TNF-α, it is possible that HLDF-6 blocks transduction of the apoptotic signal at one of the stages of the mitochondrial pathway.

For subsequent testing of the hypothesis that HLDF-6 inhibits apoptotic signal transduction at one of stages of the mitochondrial pathway, we investigated the effect of HLDF-6 peptide on HL-60 cell death induced by C_2-ceramide. The latter is an analog of natural ceramide, a highly specific and key mediator of TNF-induced apoptotic cell death. Mitochondria are the main target of ceramide [49, 50]. Ceramide induces two important stages typical for the mitochondrial apoptotic pathway: release of cytochrome *c* and the decrease in mitochondrial membrane potential [51, 52]. Concentration of C_2-ceramide was comparable to physiological concentrations of endogenous ceramide formed during cleavage of membrane sphingolipids by tissue TNF-activated sphingomyelinases [53]. Ceramide causes structural rearrangement of outer mitochondrial membrane lipid microdomains; this impairs the association of cytochrome *c* with anionic phospholipids followed by cytochrome *c* release and triggering a cascade of reactions that lead to apoptosis [54]. Based on the data obtained in this study and previous data on HLDF-6-induced changes in physicochemical properties of lipid membrane [17], we suggest that this hexapeptide promotes ordering of mitochondrial membrane structure and thus blocks the ceramide-induced release of cytochrome *c*. This mechanism may be responsible for the development of resistance of HL-60 cells to cytotoxic effect of TNF-α. On the other hand, the development of myeloid cell resistance to TNF-induced death may also be related to the increased biosynthesis of endogenous TNF-α [55].

The cytotoxic effect of TNF-α is accompanied by formation of reactive oxygen species, especially superoxide anion (O_2^-) [56]. In cells, O_2^- is readily degraded by several enzymes including superoxide dismutases (SOD) before its involvement into various reactions. Three SOD isoenzymes are known: cytoplasmic (SOD1) and extracellular (SOD3) enzymes (containing Cu^{2+} and Zn^{2+}) and mitochondrial (SOD2) Mn^{2+}-containing enzyme (also known as Mn-SOD) [57]. Endogenous TNF-α is involved in regulation of biosynthesis of Mn-SOD [58]. Blockade of endogenous TNF-α decreases Mn-SOD expression accompanied by subsequent cell death [41]. Taking into consideration that the presence of HLDF-6 increases TNF-mediated biosynthesis of endogenous TNF-α, it is also possible that blockade of apoptosis is related not only to direct effect of HLDF-6 peptide on the mitochondrial membrane structure, but also activation of biosynthesis of endogenous TNF-α, which regulates expression of one of the key mitochondrial antioxidant enzymes, Mn-SOD.

We do believe that our data make a significant contribution to understanding molecular and biochemical mechanisms responsible for resistance of differentiated cells to the cytotoxic effect of TNF-α. This is one of the key aspects for the development of new effective TNF-based methods for therapeutic treatment of cancer.

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