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# State of Nucleolar Proteins B23/Nucleophosmin and UBF in HeLa Cells during Apoptosis Induced by Tumor Necrosis Factor

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**Abstract**—The structural state of two major nucleolar proteins, UBF and B23/nucleophosmin (both monomeric and oligomeric forms), was for the first time established in HeLa cells treated with apoptosis inducers: tumor necrosis factor (TNF-α), emetine, and their combination. The treatment of the cells with either TNF-α or emetine did not induce apoptosis and affect the state of UBF and nucleophosmin (both monomers and oligomers). Apoptosis was rather pronounced only if HeLa cells were treated with a mixture of TNF-α and emetine. States of the UBF and B23 proteins were analyzed in samples containing 25, 45, and 100% of cells with apoptotic nuclei. It was shown by immunoblotting that TNF-α-induced apoptosis of HeLa cells was associated with proteolysis of UBF and production of a 76-kD fragment, the content of which increased in correlation with the fraction of apoptotically changed cells. The N- and C-terminal amino acid sequences of UBF and its 76-kD fragment were characterized, and the site of the apoptosis-induced specific proteolysis was identified. As differentiated from UBF, protein B23 did not undergo proteolytic degradation during the TNF-α-induced apoptosis of HeLa cells and its content was unchanged even in the cell fraction with fragmentation of virtually all nuclei. However, the ratio between the monomeric and oligomeric states of B23 protein was changed in apoptotic cells, and apoptosis-specific forms of nucleophosmin were detected.

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*Key words*: apoptosis, TNF, nucleophosmin, UBF, proteolysis, oligomers, HeLa

In addition to the long-known functions (transcription of ribosomal genes and biogenesis of ribosomes), the nucleolus has been recently shown to play an important role in the regulation of programmed cell death, or apoptosis [1-4]. Tumor cells are different from normal cells not only by hyperactivity and pleomorphism of the nucleoli [3], but also by sharply increased contents of some nucleolar proteins, such as B23/nucleophosmin, fibrillarin, and UBF (NOR-90) [4], leading to increased resistance of tumor cells to apoptosis. Various antitumor drugs, which are usually inducers of apoptosis, are located in the nucleolus and either change its structure (cause segregation of fibrillar and granular components, argentophilic proteins, promote translocation of some nucleolar proteins into the nucleoplasm), or inhibit its function [1, 2]. The nucleolus has been reported [5] to function as a stress transmitter responsible for maintaining a low level of p53 protein, the amount of which inevitably increases immediately on stress-caused destruction of the nucleolus structure and functioning.

We studied two major nucleolar proteins which are undoubtedly involved in apoptosis: B23/nucleophosmin (a factor of pre-ribosome assemblage) and UBF (the transcriptional factor of RNA polymerase I). Concentrations of these two proteins sharply increase in tumor cells, and their overexpression decreases the cell sensitivity to apoptosis-inducing factors [6]. Cytotoxicity of many apoptotic agents for tumor cells correlates with their ability to induce translocation of B23 protein from the nucleolus into the nucleoplasm [7-9] and activate translocation/proteolysis of UBF [10-13].

The translocation of nucleophosmin from the nucleolus is the initial stage of nucleolar segregation. This process is believed [3] to make the nucleolar DNA more

*Abbreviations*: DAB) 3,3′-diaminobenzidine; DAPI dihydrochloride) 4′,6-diamidino-2-phenylindole dihydrochloride; PBS) phosphate-buffered saline; TNF-α) tumor necrosis factor α; Versene solution) PBS containing 0.02% EDTA.

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vulnerable by nucleases. In fact, translocation of B23 protein anticipates such events as chromatin condensation and fragmentation of DNA and can act as a triggering mechanism for DNA repair, activation of the p53 protein gene, and induction of apoptosis. The translocated nucleophosmin is supposed to hold the p53 protein in the nucleoplasm and thus stabilize it [5]. The gene of nucleophosmin is involved in various chromosomal translocations, which produce fusion proteins. The proteins NPM-ALK, NPM-RAR, and NPM-MLF1 seem to be responsible for the arising of leukemia, and changes in B23 functions caused by generation of these molecules can result in a wrong regulation of p53 function in tumor cells [14]. Nucleophosmin has been recently reported to interact with two other proteins, suppressors of tumor growth: interferon-regulating factor-1 (IRF-1) [15] and interferon-induced RNA-dependent protein kinase (PKR) [16].

New literature data [4] have shown that apoptosis-mediated changes in the structure of nucleolar proteins, in particular UBF and B23, trigger the generation of autoimmune antibodies and cause some autoimmune diseases. But what is known about apoptosis-dependent changes in the structure of UBF and nucleophosmin? The majority of available data indicate that UBF is an early target of apoptosis, and in addition to its translocation from the nucleolus into the nucleoplasm, apoptosis-dependent proteolysis of UBF occurs [17-21]. Unfortunately, no cleavage sites of the polypeptide chain have been determined in these works, and no proteolytic fragments have been characterized. There are virtually no data on the monomeric–oligomeric state of B23 protein during apoptosis of tumor cells, and even data on changes in the concentration of this protein are very contradictory. Thus, on induction of apoptosis, some authors observed an decrease in protein B23 content associated with either a decrease in the amount of mRNA of nucleophosmin [3, 7] or with its proteolysis [2, 8, 9]. And similarly to the case of UBF, this proteolysis can depend not only on caspases [17-20], and many current studies are now searching for proteases active in the nucleus during apoptosis. In other works [1, 10, 12, 13], protein B23 content was shown to be unchanged and nucleophosmin only translocated in correlation with the apoptosis depth. However, in all these works only the monomeric form of the protein was assessed. But there are also data [4, 14, 22] that cell malignization is accompanied by structural changes in nucleophosmin resulting in appearance in cells of unique oligomeric (mainly hexameric) forms of the protein that are unusually resistant to heating and treatment with SDS. At present there are no data on changes in the oligomeric state of B23 protein during apoptosis of tumor cells associated with changes in its intracellular location and partner proteins.

The present work is the first to analyze the state of nucleophosmin (of both monomeric and oligomeric forms) and characterize on the protein level UBF and products of its hydrolysis during apoptosis induced by TNF-α/emetine in human cervical carcinoma cells (HeLa).

## MATERIALS AND METHODS

**Materials** used were as follows: 10% fetal calf serum, culture medium DMEM (HyClone, USA); penicillin, streptomycin (Gibco, Great Britain); 5-dimethylamino-1-naphthalenesulfonyl chloride (Dns-Cl) (Fluka, Switzerland); emetine, calibrating standard proteins (SDS-6H), NaCl, KCl, $Na_2HPO_4$, $KH_2PO_4$, EDTA disodium salt, acetonitrile, 3,3′-diaminobenzidine (DAB), goat antibodies to mouse IgG and IgM (H + L) conjugated with horseradish peroxidase, sheep antibodies to human IgG conjugated with horseradish peroxidase, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI dihydrochloride), paraformaldehyde, BSA, thymidine, PMSF-treated carboxypeptidase A (cpA) from bovine pancreas (with activity of 50 U/mg protein), polyvinylpyrrolidone-40 (PVP-40), puromycin (Sigma, USA). Recombinant human TNF-α was kindly presented by the Laboratory of Protein Engineering, Institute of Bioorganic Chemistry, Russian Academy of Sciences; reagents for electrophoresis, dry defatted milk, Tris were from Bio-Rad (USA). SDS (Bio-Rad) was twice recrystallized from 95% methanol; Immobilon NC and Immobilon P membranes with pores of 0.45 μm diameter were from Millipore (USA); monoclonal antibodies to B23 protein (3C9) were prepared in the Hematology Research Center, Russian Academy of Medical Sciences (RAMS); autoimmune serum from a patient with systemic sclerodermia was obtained in the Institute of Rheumatology, RAMS. Triton X-100 and dimethylsulfoxide (DMSO) were from ICN (USA); Mowiol was from Hoechst (USA). Other reagents were of domestic production, of chemical purity or special purity qualification.

**Cell cultures.** Human cell cultures HeLa and HeLa-Bcl-2 obtained in the Institute of Poliomyelitis and Viral Encephalitis, RAMS, by transfection of HeLa cells with the pLPS-*bcl-2* vector were used. The cells were grown on DMEM medium supplemented with 10% fetal calf serum, penicillin (10 μg/ml), and streptomycin (10 μg/ml), at 37°C in the presence of 5% $CO_2$. In the case of HeLa-Bcl-2 cells, the medium also contained puromycin (1 μg/ml). For experiments, the cells were grown in culture flasks or on cover slips.

**Induction of apoptosis.** Experiments were performed on the day after replanting, in the exponential growth phase of the cells. The cells were incubated in the presence of the protein synthesis inhibitor emetine (1 μg/ml), or tumor necrosis factor-α (TNF-α) (10 ng/ml), or their mixture at 37°C for 2 and 6 h. The control cells were incubated under the same conditions but without emetine

and TNF-α. The quantity of apoptotic cells was counted in three randomly chosen microscopic fields with a 40× objective before and after exposition to the corresponding agents. The cells starting apoptosis were identified visually with a light microscope and by staining with DAPI by the presence of characteristic morphological changes, such as vesicular structures on the membranes (blebbing), nuclear fragmentation, and appearance of apoptotic bodies. Results of determination of the apoptotic cell percent are presented as the mean ± standard deviation, calculated for 500 cells in each of three independent experiments.

**Preparing cell suspensions.** The untreated cells were washed free from the medium in phosphate-buffered saline (PBS) (pH 7.2) containing 2.7 mM KCl, 140 mM NaCl, 8.1 mM $Na_2HPO_4$, and 1.5 mM $KH_2PO_4$. The cells were removed from the culture flask surface with Versene solution for 5 min at 37°C and precipitated by centrifugation for 10 min at 1672 rpm (500*g*) at 4°C using a 5804R centrifuge (Eppendorf, Germany). The precipitate was decanted, resuspended in PBS, and centrifuged again under the same conditions. In the precipitate resuspended in PBS, the protein concentration was determined and electrophoresis and immunochemical analysis were performed.

The cells exposed to apoptotic agents were analyzed as two fractions. The floating cell fraction was poured together with the culture medium. The culture flask was washed in PBS, and the washing solutions were combined with the floating cell fraction and centrifuged for 20 min at 1672 rpm (500*g*) at 4°C. The cells adhered to the flask surface were washed in PBS, removed with Versene solution, and centrifuged under the same conditions. Precipitates of these two fractions were washed twice in PBS and centrifuged under the same conditions. The precipitates were suspended in PBS, the protein concentration was determined, and electrophoresis and immunochemical analysis were performed.

**Staining of cells with DAPI.** Cells grown on cover slips were washed in PBS and fixed with 3% paraformaldehyde solution in PBS for 20 min at room temperature. The fixed cells were washed thrice for 5 min in PBS, stained with DAPI solution (0.1 μg/ml), and mounted into Mowiol.

After apoptosis, a part of the floating cells was washed in PBS and precipitated by centrifugation for 5 min at 2048 rpm (750*g*) at 4°C using the 5804R centrifuge. The cells remaining on the flask surface were washed in PBS, removed with Versene solution, and centrifuged under the same conditions. The resulting precipitates were supplemented each with 5 ml of 3% paraformaldehyde solution in PBS, carefully resuspended by shaking, and maintained for 20 min at room temperature. The cells were precipitated by centrifugation for 5 min at 2048 rpm (750*g*) at 4°C, and then the precipitates were washed thrice for 5 min in PBS and placed onto cover slips. The cell-carrying slips were covered with 0.1% gelatin solution in PBS and dried overnight at room temperature. Afterwards, the cells were stained with DAPI solution (0.1 μg/ml) and mounted into Mowiol.

All preparations were examined with an Axiovert 200 microscope (Carl Zeiss, Germany). The images were recorded with a 12-bit monochrome CCD $CoolSnap_{cf}$ camera (Roper Scientific, USA) and processed using the Adobe Photoshop version 7.0 program.

**Protein concentration** was determined by a modification of the Lowry method [23], with BSA as the standard.

**Preparing samples for electrophoresis.** Before being treated with lysing solutions, the cell suspensions were supplemented with PBS and glycerol to adjust the protein concentration in the sample to ~1-1.5 mg/ml and the glycerol concentration to 30%. Then the samples were treated as described in [24]: to the cell suspension buffer was added which contained SDS, 2-mercaptoethanol, and EDTA, to the concentration of 5%, 2.5%, and 1 mM, respectively. The mixture was usually maintained at 80°C for 1 min or at 100°C for 10 min (to provide for degradation of oligomeric forms of B23 protein).

**SDS-PAGE and electroblotting.** Electrophoresis was performed by the Laemmli method [25] at 12°C on gel plates of 1-1.5 mm thick, at the polyacrylamide gel concentration of 7.5, 10, or 12.5% under denaturing conditions. The proteins were concentrated at the current of 10 mA/plate and separated at 20 mA/plate. The protein bands on the gel were stained with 0.1% Coomassie G-250 solution in 10% acetic acid supplemented with 25% 2-propanol. Electrotransfer was performed onto Immobilon NC membranes for immunostaining or onto Immobilon P membranes for sequencing and hydrolysis of C-terminal amino acid residues with carboxypeptidases. The electrotransfer was performed under conditions described in [26]: 0.025 M sodium bicarbonate buffer (pH 9.0) containing 20% $CH_3OH$ and 0.1% SDS. The electrotransfer was done at 12°C and constant current of ~400 mA for 4 h, or to more efficiently transfer proteins with different molecular weights, a three-stage differentiated electrotransfer was used [24]: after immunoblotting for 3 h, the gel region containing proteins with molecular weights <60 kD and the corresponding region of the membrane were cut off, and the upper part of the gel carrying high-molecular-weight proteins was electrotransferred additionally for 1 h. Then the gel region containing proteins with molecular weights <120 kD and the corresponding region of the membrane were cut off, and the upper part of the gel with proteins of higher molecular weights was subjected to additional electrotransfer for 6-8 h (depending on the percentage and the gel thickness). The completeness of electrotransfer was monitored by staining the gels (after the blotting) with 0.1% Coomassie G-250 and marker proteins on the membranes with 0.1% Amido Black 10B solution in 45% methanol. Two-stage electrotransfer was also used in the present work: the proteins with molecular weights <60 kD (the lower region of

the gel) were electrotransferred for 3 h, and the proteins with the higher molecular weights (the upper region of the gel) were electrotransferred additionally for 12 h.

**Immunostaining.** After the electrotransfer, the Immobilon membranes were washed six times for 5 min in buffer A (50 mM Tris-HCl, 200 mM NaCl, 0.1% Triton X-100, pH 7.5) on a shaker. Then the membranes were placed in buffer A containing 5% milk and incubated for 1.5 h, and then incubated overnight with monoclonal antibodies 3C9 specific to protein B23 [27] or with autoimmune antibodies specific to UBF from a patient with systemic sclerodermia, in buffer A containing 5% milk at 4°C. Then the membranes were washed in buffer A six times for 5 min, treated again with 5% milk in buffer A for 50 min, and incubated with the appropriate secondary antibodies (goat antibodies to mouse IgG + IgM conjugated with horseradish peroxidase for B23 protein or sheep antibodies to human IgG conjugated with peroxidase for UBF) in buffer A containing 5% milk for 1.5 h. The membranes were washed six times for 5 min in buffer A and thrice for 3 min in distilled water. To stain the strips, to the membranes 25 ml of 50 mM Tris (pH 7.6) containing 12.5 mg DAB was added. Immediately before the staining 10 μl of 50% $H_2O_2$ was added. The strips were developed for 5-20 min in the dark.

**The formyl protection was removed** from proteins directly on the Immobilon P membrane as described in [24].

**Analysis of N-terminal amino acid sequence of proteins.** Automated degradation according to Edman was performed with a Procise 491 Protein Sequencing System gas phase sequencer (Applied Biosystems, USA). Phenylhydantoin derivatives of amino acids were identified with a 785 A PTH-analyzer (Applied Biosystems).

**Protein hydrolysis by carboxypeptidases and analysis of C-terminal sequence.** Proteins were hydrolyzed by carboxypeptidases directly on the Immobilon P membrane. To prevent nonspecific adsorption of carboxypeptidases, the membrane was treated with PVP-40. The Immobilon strips were cut finely and moistened with 20 μl of methanol. Excess methanol was removed with a capillary pipette. The membranes were incubated in 100 μl of 0.5% PVP-40 solution in 100 mM acetic acid for 30 min at 37°C. Excess PVP-40 was removed by five washings in Milli-Q water and two washings in 0.2 M N-ethylmorpholine acetate buffer (pH 8.5). Hydrolysis by carboxypeptidases was performed in two stages. In the first stage, the membrane was supplemented with 30 μl of 0.2 M N-ethylmorpholine acetate buffer (pH 8.3) containing 1 μg cpA. The mixture was incubated for 120 min at 37°C. In the second stage, the buffer was acidified with $CH_3COOH$ to pH 6.0, then 1 μg cpA was added, and hydrolysis was continued at 37°C for 120 min. On termination of the reaction, the supernatant was separated, and the membrane was washed successively in 0.2 M N-ethylmorpholine acetate buffer (pH 6.0), Milli-Q water, and methanol (by portions of 20 μl). All washing solutions were combined with the supernatant and evaporated. The detached amino acids were analyzed as dansyl derivatives by two-dimensional chromatography on 5 × 5 cm silica gel plates as described in [24, 28].

## RESULTS AND DISCUSSION

TNF-α is now the best-studied and most efficient physiological inducer of apoptosis in animal cells [29, 30]. Cytotoxic effects of TNF-α are balanced by defense mechanisms in some cell lines, including the HeLa tumor cells used by us; therefore, TNF-α does not affect the cell viability. Because antiapoptotic defense mechanisms depend on protein synthesis, the TNF-α-induced apoptosis can occur only in the presence of protein synthesis inhibitors [29]. We have chosen the reversible inhibitor emetine, which blocks translation and translocation [31]. We have also used HeLa-Bcl-2 cells transfected with a construct containing cDNA of the antiapoptotic protein Bcl-2.

**Preparation of apoptotic cells.** Rather pronounced apoptosis was observed in HeLa cells only in the combined presence of TNF-α and emetine (Fig. 1). In the control cell culture, the number of nuclei with morphologic signs of apoptosis was low (~1-2%). The number of such nuclei increased to 25% on exposure for 2 h to apoptosis inducers (mixture of TNF-α and emetine); these nuclei had irregular shape and distinct heterochromatin fragmentation. Nuclear fragmentation is the main sign of the cell starting apoptosis [32, 33]. After 6 h of the exposure to apoptosis inducers, the number of cells with fragmented nuclei increased to 60%. Similarly to results of work [34], in our experiments Bcl-2 had a protective effect, and the number of HeLa-Bcl-2 cells with apoptotic nuclei was comparable to that in the control. Thus, according to our findings and the published data [34-36], similar schemes of apoptosis induction by the 6-h expo-

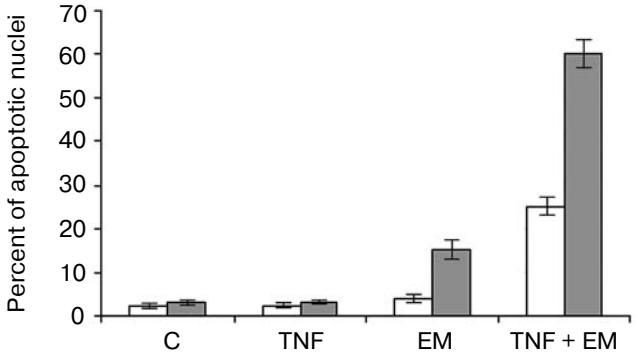


**Fig. 1.** Percent of apoptotic nuclei in HeLa cells treated with TNF-α (TNF), emetine (EM), and their mixture for 2 h (light rectangles) and 6 h (dark rectangles). C, control (untreated cells).

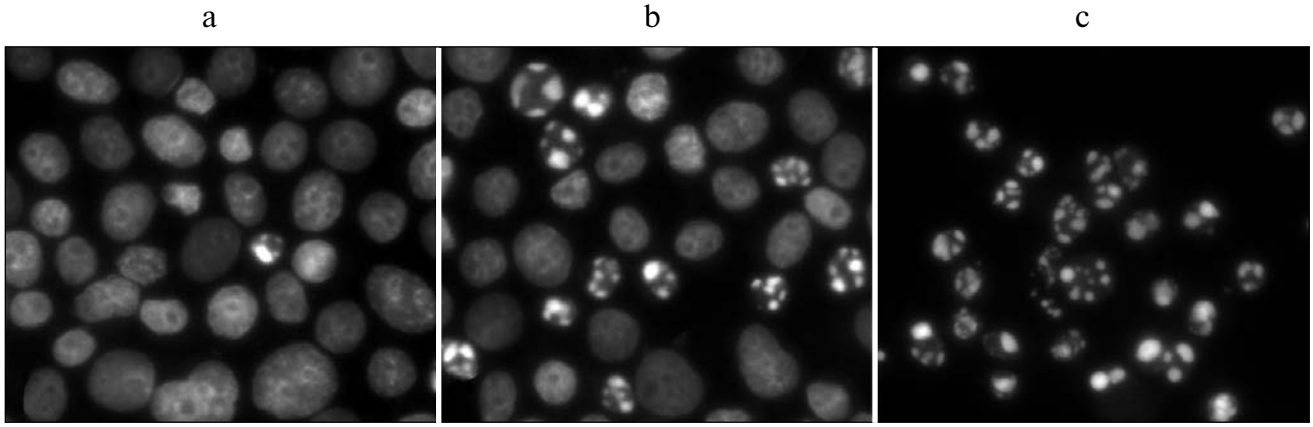


**Fig. 2.** Staining of HeLa cells with DAPI in control cells (a) and in cells incubated for 6 h in the presence of apoptosis inducers (combined emetine and TNF-α) (b, c). Two fractions of the cells were analyzed: those adhered to the surface of the culture flask (b) and those floating (c).

sure of HeLa cells to the TNF-α and emetine mixture resulted in appearance of a large number of cells with pronounced signs of apoptosis.

We found that many of those cells treated for 6 h with the TNF-α and emetine mixture lost their adhesiveness and floated into the culture medium. Therefore, we analyzed two fractions of the cells: the unfastened (floating) cells and those adhered to the culture flask surface. After the exposure to apoptosis inducers for 6 h, 45% of the adhered cells (Fig. 2b) and 100% of the floating cells had fragmented nuclei (Fig. 2c).

Apoptosis-induced changes in the protein structure are usually studied in the total population of cells exposed to apoptotic agents, although such a population includes both apoptotic and nonapoptotic cells. We know only two works [19, 37], which analyzed pure populations of completely apoptotic cells. In work [19], the fraction of apoptotic cells was isolated by centrifugation in a Percoll density gradient from a Jurkat suspension cell culture of T-lymphoblasts exposed to the apoptosis inducer EGTA (or lovastatine). In work [37], the fraction of apoptotic cells of the aorta endothelium was used which unfastened from the monolayer on the treatment with a combination of a cytokine TNF and the protein synthesis inhibitor cycloheximide. The apoptotic fractions were analyzed by two-dimensional electrophoresis, and the protein composition of the apoptotic and control cells occurred to be strikingly alike, changes being recorded only in the phosphorylation level (usually a decrease) [37] and in the contents of some proteins [19].

Comparison of electrophoregrams of the control cells and the cells treated with apoptotic agents (TNF-α, emetine, and their mixture) led to the following conclusions. First, the set and intensity of the major protein bands were comparable in all lanes (Fig. 3a), which is consistent with the data of studies [19, 37]. Second, as distinct from the control cells and apoptotic cells with retained adhesiveness, in the electrophoregram of the floating cell fraction the band corresponding to 66-kD proteins was markedly strengthened (Fig. 3a, lane *6*), and we believed it to represent BSA [38]. The fraction of apoptotic cells was supposed to be capable of increased uptake and/or sorption of BSA from the culture medium. The significance and mechanism of this phenomenon are still unclear.

In some works [10-13], UBF was shown to be an early target of the apoptosis-induced proteolysis, which depended on both cell type and apoptosis inducers. We decided to assess the behavior of UBF during apoptosis of HeLa cells induced by TNF-α/emetine.

**Changes in UBF during apoptosis.** By Western-blot analysis with autoimmune antibodies to UBF from a patient with systemic sclerodermia, a partial proteolysis of UBF was revealed resulting in a 76-kD fragment even after 2-h treatment with the TNF-α and emetine mixture, when the number of cells with apoptotic nuclei was ~25% (Fig. 4, lane *2*). Prolongation of the treatment time to 6 h increased the number of apoptotic cells to 60% and enhanced the proteolysis of UBF associated with production of this fragment (Fig. 4, lanes *3* and *4*). The cleavage was most pronounced in the floating cells in the terminal stages of apoptosis (Fig. 4, lane *4*). The electrophoretic separation in 12.5% polyacrylamide gel ensured screening of polypeptides with molecular weight from 12 to 240 kD. However, in addition to the duplicate of bands corresponding to two full-size isoforms of UBF (~96-98 kD), the only stable proteolytic fragment of ~76 kD was detected in the immunoblot (Fig. 4), and the content of this fragment increased in correlation with a decrease in the content of full-size UBF during apoptosis. Thus, the TNF-α-induced apoptosis in HeLa cells was associated with a characteristic proteolytic fragmentation of UBF, and even at early stages of apoptosis the UBF protein was not only translocated from the nucleolus into the

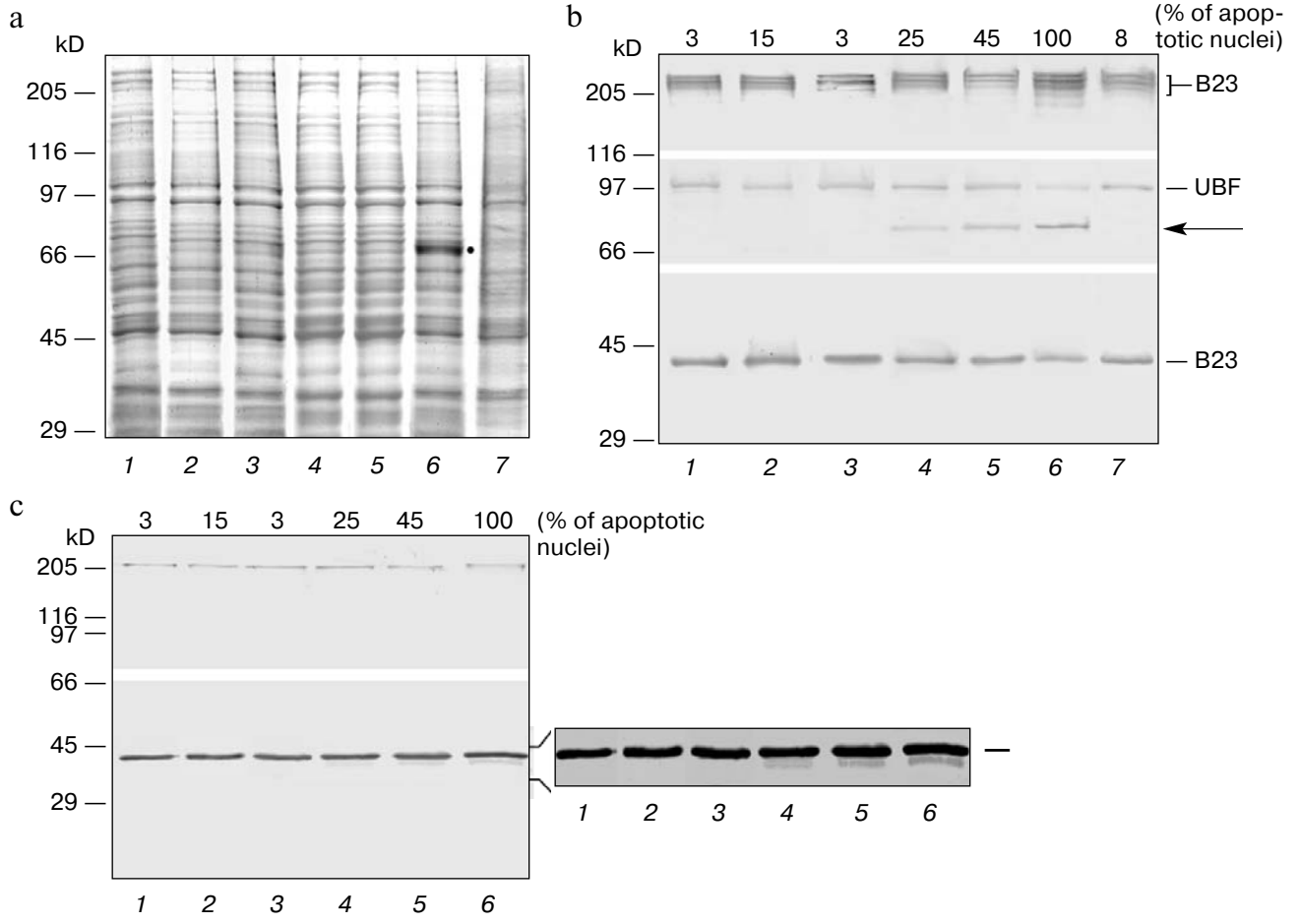


**Fig. 3.** Electrophoregram (a) and immunoblots (b, c). Immunochemical analysis of the B23 and UBF proteins in HeLa cells in apoptosis induced by TNF-α, emetine, and their mixture. The electrophoresis was performed by the Laemmli method in 7.5% (a, b) and 10% (c) polyacrylamide gel with SDS. Samples used: lysates of control HeLa cells (*1*) and of HeLa cells treated with emetine (*2*), TNF-α (*3*) for 6 h, and with the emetine and TNF-α mixture for 2 h (*4*) or 6 h (cells adhered on the culture flask surface (*5*); floating cells (*6*)), and also of HeLa-Bcl-2 cells treated with the emetine and TNF-α mixture for 6 h (*7*). The samples for electrophoresis were treated as described in "Materials and Methods" and heated for 1 min at 80°C (a, b) or for 10 min at 100°C (c). The proteins were electrotransferred onto an Immobilon membrane by three (b) or two stages (c). The Immobilon NC membranes were immunostained with monoclonal antibodies 3C9 specific to B23 protein (b, c) or autoimmune antibodies to UBF (b). Intact proteins are indicated by the line, the proteolytic fragment of UBF is shown by the arrow. At the top of the figure, the percentage of cells with apoptotic nuclei is indicated (b, c). Positions of the marker proteins are shown to the left.

nucleoplasm (as shown in [36]) but also degraded by proteolysis.

The apoptosis-induced proteolysis of UBF was also observed in HL-60 cells treated with camptothecin [12] and in Jurkat cells treated with CD-95 (Fas/APO-1) [10, 11, 13], and the fragmentation types were different. But in neither of these works were the regions of UBF proteolysis characterized.

The amino acid sequence of UBF has been determined based on analysis of the cDNA structure, but there are no data concerning N- and C-terminal processing. To characterize the structure of the protein and its proteolytic fragment, we cut off two protein strips from the Immobilon P membrane; one of these strips corresponded to the full-size protein UBF (Fig. 4, lane *1*) and the other to its 76-kD proteolytic fragment (Fig. 4, lane *4*). The amino acid sequence of UBF was determined beginning from the $Met^{1}$ residue (according to the structure derived from the cDNA sequence) (Fig. 5). Analysis of the C-terminal sequences of UBF and its proteolytic fragment using carboxypeptidase A allowed us not only to determine the C-terminal sequence of UBF, but also establish that the 76-kD fragment was the C-terminal fragment of the protein (Fig. 5). Sequencing of the N-terminal amino acid sequence of the 76-kD fragment of UBF (Fig. 5) revealed the region of the apoptosis-specific proteolysis. The sequence of its twelve N-terminal amino acids coincided with the amino acid sequence of UBF: $^{186}$LIQNAKKSDIPE$^{197}$. Proteolysis of the protein in this region of the polypeptide chain resulted in detach-

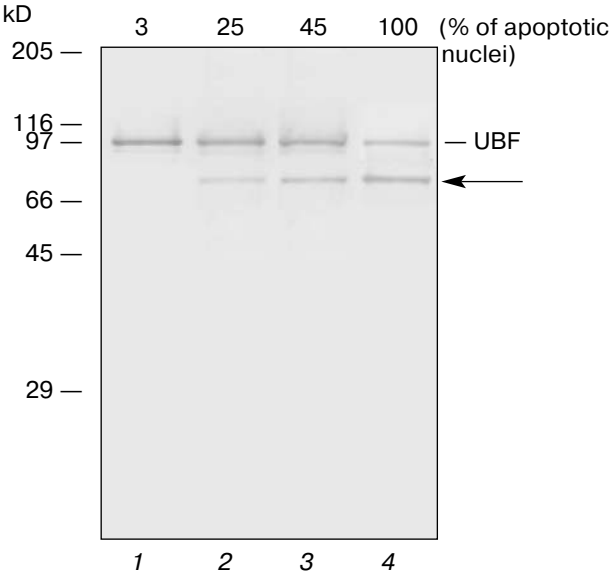


**Fig. 4.** Immunochemical analysis of UBF in HeLa cells in apoptosis induced by the TNF-α and emetine mixture. The immunoblot with autoimmune antibodies to UBF from lysates of the control HeLa cells (*1*) and HeLa cells treated with the emetine and TNF-α mixture for 2 h (*2*) and 6 h (cells adhered on the culture flask surface (*3*); floating cells (*4*)). The electrophoresis was performed by the Laemmli method in 12.5% polyacrylamide gel with SDS. The proteins were electrotransferred onto an Immobilon membrane during 4 h. The intact UBF protein is shown by the line, the product of its proteolysis is indicated by the arrow. At the top of the figure, the percentage of cells with apoptotic nuclei is indicated. Positions of the marker proteins are shown to the left.

ment of the N-terminal fragment, which contained a domain extremely important for both UBF dimerization and its functioning as a transcription factor and one of six HMG-boxes [40].

Unfortunately, we cannot test the N-terminal fragment of UBF (and possible products of its proteolysis) because of limitations associated with the immunodetection method (this fragment seems to lack the antigenic determinant). Nevertheless, analysis of the specific apoptosis-induced proteolytic fragmentation in the UBF polypeptide chain region resulting in the stable 76-kD fragment seems very informative for the subsequent identification of the protease responsible for hydrolysis of nuclear–nucleolar proteins during apoptosis. It is difficult to identify the protease involved in the apoptosis-specific proteolysis of UBF. Although the proteolysis region $^{182}$DHPD↓$^{186}$L corresponds, for instance, to the specificity of caspases 2 and 3 (DXXD↓), we could not specify the type of caspase responsible for proteolysis of UBF based on literature data on the substrate specificity of other caspases. In particular, this is because of contradictions in data on the substrate specificity of other caspases. Moreover, the substrate specificities of some caspases (e.g., caspase-10, -13) are not yet established [41, 42].

**Changes in protein B23/nucleophosmin during apoptosis.** It is interesting to assess the state of the whole complex of nucleolar proteins during apoptotic death of the cells. We decided to study apoptosis-induced changes in the structure of another major nucleolar protein, nucleophosmin, which is different from UBF in intranucleolar location, functions, and also in the translocation manner from the nucleolus into the nucleoplasm during apoptosis of HeLa cells induced by TNF-α [36].

It was especially interesting to assess the state of B23 protein during apoptosis because only its monomeric form had been characterized in the majority of works. But analysis of only monomeric forms is insufficient, first, because both nucleophosmin forms can *in vitro* and *in vivo* produce oligomers (mainly ~210-230 kD hexamers) which can be both homo- and heterooligomers [43, 44]. Production of oligomers, and mainly hexamers, is also specific for expressed protein B23, as shown by gel filtration, gel electrophoresis [45], and sedimentation analysis [46]. Second, tumor cells have been shown to have unique

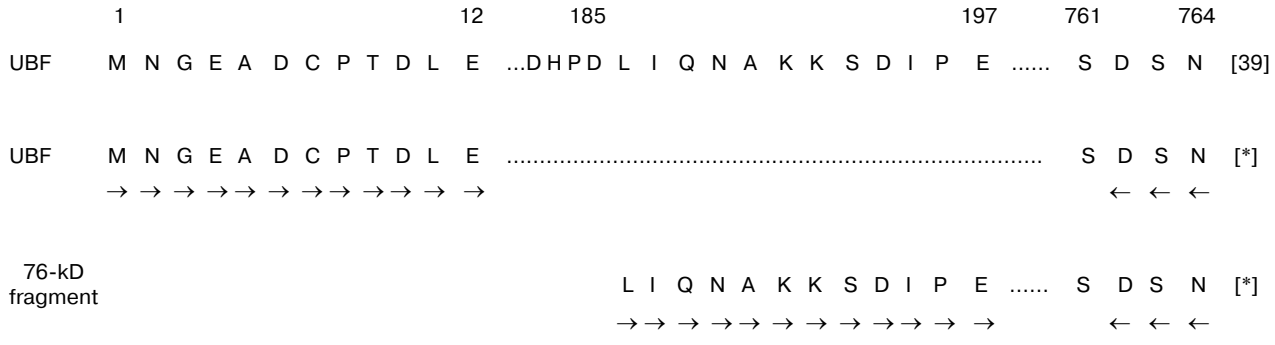


**Fig. 5.** Analysis of the N- and C-terminal amino acid sequences of UBF and its 76-kD proteolytic fragment. At the top of the scheme, the amino acid sequences of human UBF fragments are presented [39]. The asterisk indicates the N- and C-amino acid sequences of the UBF protein and its proteolytic fragment determined by us based on results of sequencing and hydrolysis of amino acids cleaved by cpA.

hexamers consisting only of nucleophosmin isoforms [24, 47] with an unusually high resistance to treatment with SDS and heating [4, 24, 47-49]. However, there are no data on the monomeric–oligomeric state of B23 protein during apoptosis. The absence of such data is due, in particular, to the absence of methods to concurrently detect and quantitatively evaluate both oligomeric and monomeric forms of B23 protein.

We developed a scheme [24] for concurrent detection and semiquantitative evaluation of monomeric and oligomeric forms of B23 protein. This scheme includes some modifications at the stages of preparing samples for electrophoresis (compared to the Laemmli method) and electroblotting. Differential electrotransfer of proteins onto Immobilon membranes allowed us to quantitatively transfer proteins of a wide range of molecular weights. Using this scheme, we succeeded not only in concurrently detecting the earlier described monomeric and oligomeric forms of B23 protein in lysates of HeLa cells, but also in finding three SDS-resistant oligomeric forms. In these oligomers, we identified two isoforms (B23.1 and B23.2) of nucleophosmin, and the isoform B23.2 was identified in human tumor cells for the first time [24]. We used this new approach including the differential multistage electrotransfer to compare the states of B23/nucleophosmin and UBF proteins during apoptosis of HeLa cells.

In the first stage, the proteins were separated in 7.5% polyacrylamide gel in the presence of SDS. The absence of protein bands on the gel (after blotting) and the identical staining of the marker protein bands on the gel (before blotting) and on the membrane (after blotting) confirmed the complete transfer of proteins with molecular weights from 29 to 240 kD. We used for immunodetection (Fig. 3b) monoclonal antibodies to B23 protein (upper and lower parts of the membrane) and autoimmune antibodies to UBF protein from a patient with systemic scleroderma (middle part of the membrane). Immunoblots of the proteins from HeLa cells treated only by emetine or TNF-α (lanes *2* and *3*) and from HeLa-Bcl-2 cells (lane *7*) treated with the TNF-α and emetine mixture were identical to immunoblots of the proteins from the control cells (lane *1*): bands could be detected which corresponded to the full-size UBF, and monomeric and three oligomeric forms of B23 protein. Analysis of the immunoblots allowed us to show for the first time that in the fractions with a high percentage of apoptotic nuclei including the cells in its terminal stages (with obvious proteolysis of UBF) SDS-resistant oligomeric forms of nucleophosmin were retained.

Moreover, we detected in apoptotic cells some specific features of nucleophosmin. Thus, in lysates from the cells in the terminal stages of apoptosis (Fig. 3b, lane *6*) the ratio of monomeric and oligomeric forms of B23 protein was changed: the amount of monomers was decreased and two additional (compared to the lysates from control cells) bands appeared in the zone of oligomers with lower (~180-200 kD) molecular weight (although the same amounts of total cell protein were placed onto the lanes).

In some works, apoptosis-dependent decrease in nucleophosmin amount was shown (on assessment only of its monomeric form) associated with caspase-mediated [2, 8, 9] or caspase-independent proteolysis [4, 17-19] resulting in a ~21-22 kD proteolysis-resistant fragment [4, 8, 9, 17, 18]. By immunochemical analysis of proteins of the floating apoptotic cells (after their separation in 15% polyacrylamide gel) we demonstrated [38] the absence of protein B23 cleavage products of >6 kD. However, this finding did not allow us to completely exclude proteolysis of nucleophosmin, because its hydrolysis could also produce antigen-carrying peptides with lower molecular weight, and the structure of the antigenic determinant could be disturbed.

We suggested that the decrease in the content of monomeric B23 during apoptosis could be caused not only by proteolysis but also by displacement of the monomer/oligomer equilibrium towards production of oligomers. Therefore, we decided to elucidate whether the apoptosis-induced decrease in protein B23 content was caused by the displacement of the monomer/oligomer equilibrium to oligomers.

Increasing the time of thermal treatment of samples for electrophoresis is known to promote degradation of oligomeric forms of B23 protein [45, 46]. We used thermal treatment conditions developed by us earlier [38] (10 min, 100°C) to destroy oligomeric forms of nucleophosmin. The proteins were separated by electrophoresis in 10% polyacrylamide gel (the optimum conditions for separation of nucleophosmin monomers). Under these conditions, oligomeric forms of nucleophosmin were virtually completely destroyed (Fig. 3c) in both the control and treated cells. However, even in cells in the terminal stages of apoptosis the intensity of the nucleophosmin band in the zone of monomers was comparable to its intensity in the control cells (Fig. 3c, lanes *1* and *6*). These data combined with our earlier results [38] make unlikely the proteolysis of nucleophosmin during the apoptosis of HeLa cells induced by the TNF-α/emetine mixture. The findings of the present work are consistent with data on camptothecin-induced apoptosis of HeLa cells [12] and apoptosis of Jurkat cells treated with CD-95 [10, 11, 13]. In these works the cleavage of UBF and the absence of the B23 proteolysis were recorded, and the protein B23 content was unchanged even on complete proteolysis of UBF [13].

However, in apoptotic cells with pronounced proteolysis of UBF (Fig. 3b, lanes *4-6*) degradation of nucleophosmin oligomers was accompanied by appearance on immunoblots of an additional band with higher electrophoretic mobility in the zone of monomers (Fig. 3c, lanes *4-6*). The intensity of this band was low but increas-

ing proportionally to the fraction of the cells with apoptotic nuclei, and this seemed to indicate the appearance of an apoptosis-specific form of B23 protein. The appearance of this form of nucleophosmin could be associated with chemical modifications of the polypeptide chain (acetylation, phosphorylation, etc.), and N/C-terminal processing. Thus, it was found that the phosphorylation degree of B23 protein decreased during apoptosis [17], the phosphorylated and dephosphorylated forms of the protein B23.1 appeared on immunoblots as a pair of bands, and differed in intranuclear location and properties [44]. This new apoptosis-specific form of nucleophosmin was not characterized in the present work (because of its low content), but it is clearly of interest. Possibly, just this new form of B23 protein is responsible for changes in the ratio of monomeric and oligomeric forms and appearance of additional oligomers of B23 protein in the fraction of apoptotic cells, especially in the fraction of fully apoptotic ones where its content is highest.

Thus, we are the first to show that the cascade of apoptotic reactions induced by TNF-α in HeLa cells is accompanied not only by proteolysis of proteins (as in the case of UBF) but also fine structural changes resulting in new, apoptosis-specific forms of proteins (as shown for nucleophosmin), without significant changes in the total content of the protein. It can be suggested that in some proteins present as several isoforms and prone to formation of homo- and heterooligomers with different location and properties (as in the case of nucleophosmin) structural changes can concern not the total protein fraction but its separate structural forms.

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