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Decreased Thymosin β_4 in Apoptosis Induced by a Variety of Antitumor Drugs

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ABSTRACT. As many antitumor drugs can kill tumors through the induction of apoptosis, the effect of these drugs presumably would be enhanced if they were used in combination with other drugs that interact with apoptotic processes. To clarify the biological events involved in the induction of apoptosis, we examined changes in the proteins associated with induction of apoptosis by antitumor drugs. When Molt-4 cells were exposed to the antitumor drugs etoposide, meso-2,3-bis(3,5-dioxopiperazine-1-yl)butane (ICRF-193), and neocarzinostatin, they exhibited apoptotic cell death as determined by flow cytometry using fluorescein isothiocyanate (FITC)-labeled annexin V staining of phosphatidylserine on membranes and detection of hypodiploid cells. Following the induction of apoptosis, a low molecular weight protein that was identified to be thymosin β_4 by HPLC analysis was commonly decreased, and the morphology of actin filaments changed into clump formations. These results suggest that decreased thymosin β_4 is involved in the induction of apoptosis by antitumor drugs. BIOCHEM PHARMACOL **57**;10:1105–1111, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. apoptosis; antitumor drugs; thymosin β_4; actin; Molt-4

Apoptosis is an active cell death mechanism for negative selection of cells that become unnecessary for maintaining a healthy life in the development of cell lineages in embryogenesis and in adult tissues [1]. Tumor cells that escape from apoptosis and are allowed to develop at an early stage frequently result in the appearance of cells resistant to anti-cancer drugs [2]. Many tumor cells with a defect or alteration of tumor suppressors such as p53 and RB are resistant to the induction of apoptosis by DNA-damaging agents [3, 4].

Apoptosis is characterized by abnormal chromosome condensation and fragmentation, production of apoptotic bodies, reduced membrane electric potential in the mitochondria, and exposure of phosphatidylserine on the outer membrane surface [5]. These biological events associated with apoptosis are triggered by stimuli such as cytokines or hormones, growth factor withdrawal, and DNA-damaging agents. They are processed sequentially by the control of proteases called caspases, and this finally leads to DNA fragmentation and chromosome condensation [6]. Since *ced-3*, an effector gene of nematode cell death, was found to be homologous to interleukin-1β-converting enzyme (ICE, caspase I), at least nine caspase homologues have been isolated [7]. Release of cytochrome *c* from the mitochondria or activation of protein kinases such as protein kinase C and MAP¶ kinase are also involved in the apoptotic process [8].

Since many antitumor drugs kill tumors through the induction of apoptosis, synergistic elevation of the apoptosis induced should be achieved by combination chemotherapy. Therefore, to improve current therapy, we examined the common events associated with antitumor drug-induced apoptosis.

MATERIALS AND METHODS

Cell Culture

Molt-4 cells, a human lymphocyte cell line, were cultured in RPMI-1640 medium containing 10% calf serum under a humidified atmosphere of 5% CO_2 in air.

Cell Proliferation

Cell proliferation was evaluated by measuring the fluorescence intensity in the presence of Alamar Blue (Wako Pure Chemical Industries) [9]. Cells were seeded in 96-well multidishes (Costar Corp.) at a density of 1.5×10^4

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¶ *Abbreviations:* MAP, mitogen-activated protein; PI, propidium iodide; FITC, fluorescein isothiocyanate; HFS, hypotonic fluorochrome solution; and RT-PCR, reverse transcriptase–polymerase chain reaction.

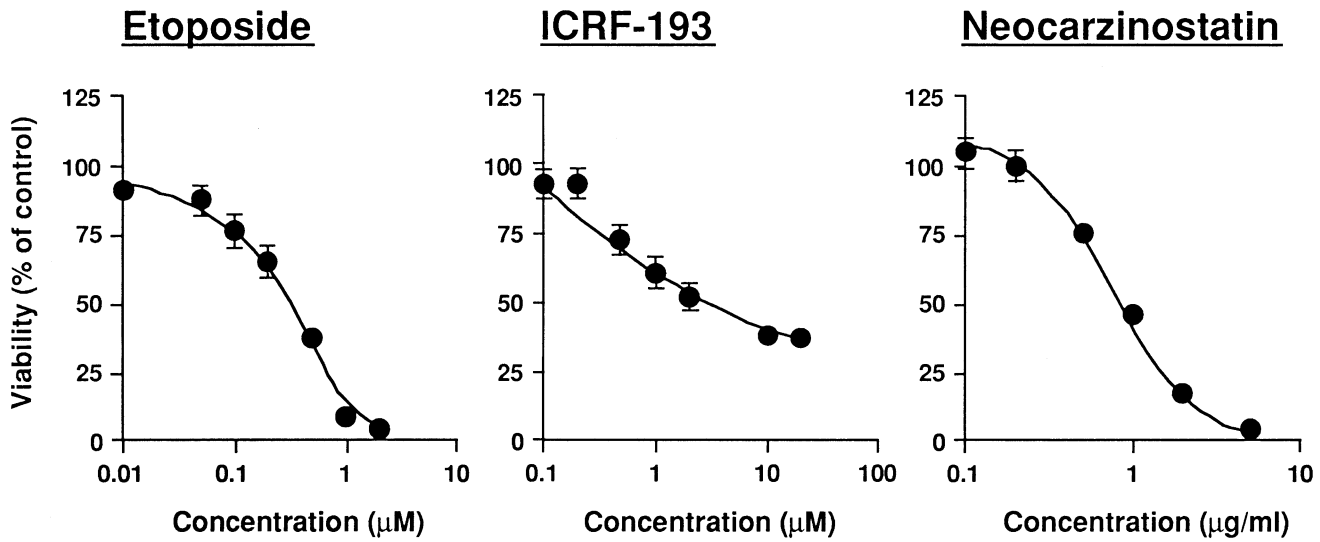

FIG. 1. Effect of antitumor drugs on the growth of Molt-4 cells. The cells (1.5×10^4) were treated with increasing concentrations of the antitumor drugs, and cell growth was examined by the Alamar Blue assay. Values represent the means ± SD from five incubations.

cells/well in culture medium and incubated overnight before being treated with the various drugs for 24 hr. To each well, 20 μL of Alamar Blue was added, and the plates were preincubated for 4 hr. Fluorescence intensity was measured using a Cytofluor 2350 with excitation at 530 nm and emission at 590 nm.

Analysis of Apoptosis by Annexin Staining

Apoptosis was determined by an apoptosis detection kit (R & D Systems). Briefly, after drug treatment, Molt-4 cells were collected and washed twice with PBS and once with a binding buffer (HEPES-buffered saline solution supplemented with 2.5 mM calcium chloride). The cells were dissolved in the binding buffer at a concentration of 1×10^5 cells/mL. PI and FITC-labeled annexin V were added to the solution, and the samples were incubated for 15 min before being analyzed within 1 hr on a FACScan (Becton-Dickinson), using LYSYS-2.

Analysis of Apoptosis by Measuring the DNA Content Per Cell

Cells were washed with PBS, fixed with 3 vol. of 70% ethanol, and incubated for 24 hr at 4°. After washing twice with HFS, the cells were suspended with HFS containing PI, sodium citrate, and Triton X-100 at final concentrations of 50 μg/mL, 0.1%, and 0.1%, respectively, and then incubated for 30 min at room temperature [10]. The samples were washed with HFS and analyzed on a FACScan.

HPLC of Intracellular Proteins

Molt-4 cells (80% confluent) in 100-mm culture dishes were exposed to various drugs for 24 hr. They were washed with cold PBS, sonicated in 20 mM Tris–HCl buffer, pH 7.4, and centrifuged at 105,000 *g* for 60 min at 4°. The resulting supernatant was subjected to reversed-phase HPLC (μBondasphere, 5 μm, C18, 300 Å, 3.9 × 150 mm) using an acetonitrile gradient at a flow rate of 1 mL/min. Proteins were monitored by measuring the absorbance at 210 nm.

Analysis of N-Terminal Amino Acid Sequence

After HPLC, the protein collected at each peak was evaporated to dryness, and digested with *Achromobacter* protease I (EC 3.4.21.50). After separation of the peptides by C18 reversed-phase HPLC, their amino acid sequences were determined with an Applied Biosystems Protein Sequencer model 473A.

Immunofluorescence

Molt-4 cells were exposed to various drugs for 24 hr, and rinsed twice with PBS. They were suspended in PBS at 1×10^6 cells/mL and plated onto poly-L-lysine-coated cover slips. After 30 min, they were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 30 min. The fixed cells were made permeable by treatment with Triton X-100 in PBS for 5 min at room temperature, after which they were incubated for 30 min at room temperature with 2% BSA in PBS. Then the cells were incubated for 1 hr with anti-thymosin β_4 antibody. The polyclonal antibody against thymosin β_4 was generated by immunizing rabbits with a synthetic peptide corresponding to the 11 carboxy terminal amino acids [11]. After incubation, the cells were rinsed three times with PBS and stained with FITC-conjugated goat anti-rabbit IgG for 1 hr in the dark at room temperature. In some cases, the cells were double stained with 0.07 μM rhodamine-phalloidin (Sigma) to detect the actin filaments.

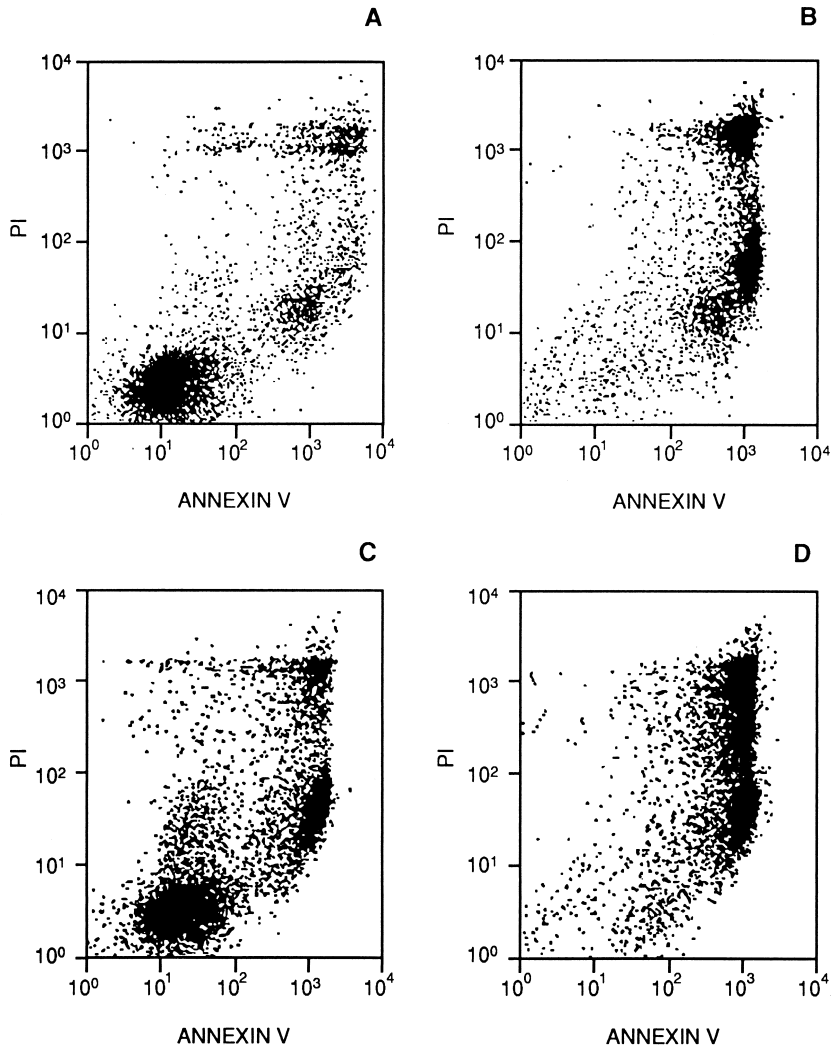

FIG. 2. Flow cytometric analysis of cells stained with annexin V-fluorescein and PI. Untreated Molt-4 cells (A). Cells were cultured in the presence of 1 μM etoposide (B), 20 μM ICRF-193 (C), or 5 μg/mL of neocarzinostatin (D), respectively, for 24 hr prior to being stained. Similar results were observed in three independent experiments.

RESULTS

Effect of Antitumor Drugs on Cell Growth in Molt-4 Cells

The antitumor drugs etoposide and meso-2,3-bis(3,5-dioxopiperazine-1-yl)butane (ICRF-193), which are inhibitors of DNA topoisomerase II, and neocarzinostatin, which is a DNA-damaging agent, were used in this study [12–15]. Etoposide is a cleavable complex-forming type of inhibitor, whereas ICRF-193 is a catalytic inhibitor that does not form a cleavable complex between DNA and topoisomerase II. Neocarzinostatin directly causes DNA breaks and release of nucleic acid bases, producing apurinic and apyrimidinic sites [15, 16].

The effect of these antitumor drugs on cell viability was examined by the measurement of fluorescence intensity in the presence of Alamar Blue. When Molt-4 cells were exposed to these drugs, cell viability was reduced in a concentration-dependent manner (Fig. 1). Etoposide was the most effective at cell killing as a function of concentration, and ICRF-193 was the least effective.

Drug-Induced Apoptosis

FITC-labeled annexin V has been used to detect apoptotic cells, since annexin binds to phosphatidylserine exposed on the outer membrane in apoptotic cells [17, 18]. To distinguish between apoptosis and necrosis, cells were double-stained with PI since apoptotic cells exclude dyes such as trypan blue or PI, while necrotic cells do not. The data in Fig. 2 show PI staining on the ordinate and annexin V staining on the abscissa after exposure of Molt-4 cells to etoposide, ICRF-193, or neocarzinostatin. The lower left staining population of cells represents viable cells that excluded PI and did not bind annexin. The upper left population shows necrotic cells, which cannot exclude PI and were not stained with FITC-labeled annexin V. When the cells were exposed to the drugs, the lower right population of cells, which represents the apoptotic cells with positive annexin V staining, was increased.

To confirm that these inviable cells were undergoing apoptosis, the number of hypodiploid cells (apoptotic cells) was examined by flow cytometry. Hypodiploid cells can be

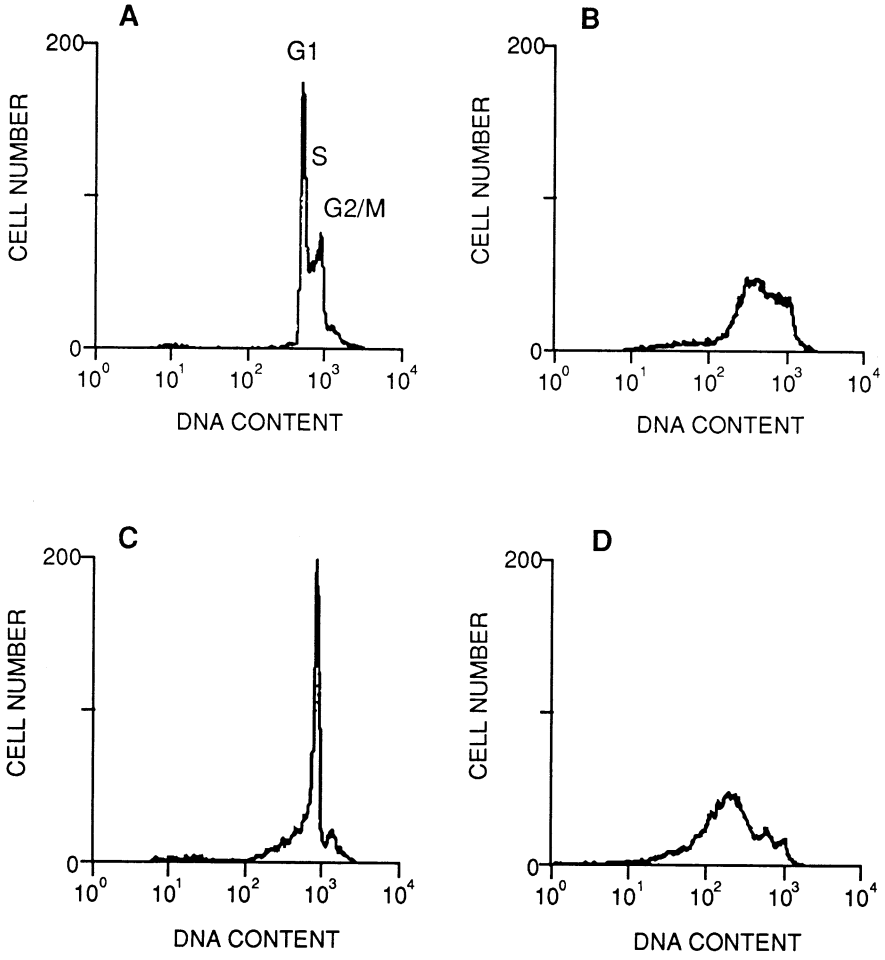

FIG. 3. DNA fluorescence histograms of PI-stained Molt-4 cells. The conditions of drug treatment were the same as in Fig. 2 except for the drug concentrations, which were 1 μM, 10 μM, and 3 μg/mL for etoposide (B), ICRF-193 (C), and neocarzinostatin (D), respectively. Panel A shows untreated cells. Similar results were observed in three independent experiments.

induced by agents that cause apoptosis but not necrosis, and they contain less DNA than normal G_1 cells [10, 19]. As shown in Fig. 3A, cells exhibited typical cell populations that corresponded to cells in G_1, S, and G_2–M phase. After a 24-hr incubation with the drugs, cellular DNA content was reduced (Fig. 3, B–D), which means that the drugs induced apoptosis. As shown in Fig. 1, ICRF-193 induced a lower level of apoptosis than the other drugs in both the annexin staining and hypodiploidy induction assays.

Decrease of Intracellular Proteins and Their Identification in Drug-Induced Apoptotic Cells

Apoptosis is characterized by sequentially occurring events such as DNA fragmentation, abnormal chromosome condensation, reduced membrane electric potential in the mitochondria, and formation of apoptotic bodies [5]. To investigate more closely the process of apoptosis induction, we tried to identify the proteins involved in apoptosis that changed quantitatively or qualitatively. The changes in intracellular proteins during apoptosis of drug-treated Molt-4 cells were examined by reversed-phase HPLC analysis. When the extracts from drug-treated cells were compared with those of non-treated cells, a main protein peak (peak 1) was reduced by all three antitumor drugs, while peak 2 did not change significantly (Fig. 4). To identify the decreased protein, the protein from the peak 1 fraction was purified and digested with *Achromobacter* protease I. The peptides generated were then analyzed by amino acid sequencer. The proteins corresponding to peaks 1 and 2 on the HPLC chromatogram were identified as thymosin β_4 and thymosin $\beta_{10,}$ respectively (Fig. 5).

Morphological Change of Actin Microfilaments in Drug-Induced Apoptotic Cells

In these drug-induced apoptotic cells, the amount of thymosin β_4, which is a major G-actin sequestering protein, was reduced. Thus, we next examined whether the morphology of the actin network was changed. Molt-4 cells were plated onto poly-L-lysine coated cover slips and stained with phalloidin and anti-thymosin β_4 antibody. In Molt-4 cells, the network of actin fibers was not well organized and showed a rather diffused distribution. However, when the cells were exposed to the drugs for 24 hr, there was an alteration in the actin structures, and clumps of actin filaments were observed (Fig. 6). The distribution of thymosin β_4 was also disorganized in all treated cells, compared with the control cells (Fig. 6). The change in the

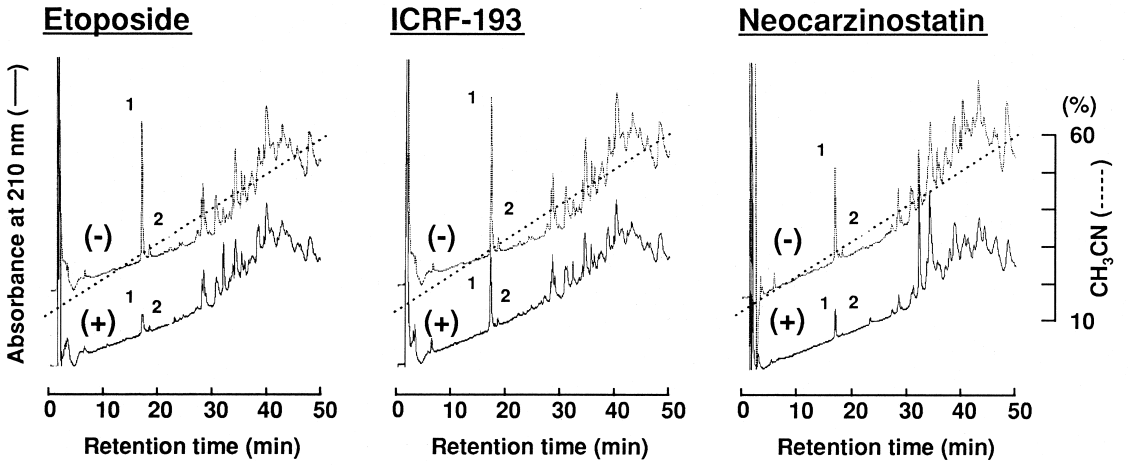

FIG. 4. Reversed-phase HPLC profile of the intracellular proteins in Molt-4 cells. The cells were treated with the antitumor drugs for 24 hr and then subjected to HPLC as described in Materials and Methods. The treatment conditions were the same as in Fig. 2. Similar results were observed in three independent experiments. The proteins corresponding to peaks 1 and 2 were identified as thymosin β_4 and β_{10}, respectively.

intracellular distribution of thymosin and the actin fiber was least when the cells were treated with ICRF-193.

DISCUSSION

In the present study, we showed that a low molecular weight protein, thymosin β_4, is decreased during apoptosis induced by various antitumor drugs. The drugs used are different types of inhibitors. Etoposide and ICRF-193 are inhibitors of DNA topoisomerase II, but the former is a cleavable complex-stabilizing type of inhibitor, while the latter is a non-cleavable type [12–14]. Neocarzinostatin directly causes DNA strand breaks [15, 16]. Thus, DNA breaks are not a common biological event induced by the three drugs. The actin cytoskeleton is implicated in many cellular functions, such as motility, cell growth, transformation, and differentiation [20–22]. Thymosin β_4, one of the G-actin binding proteins (which also include profilin and actin depolymerizing factor), controls cytoskeleton organization through regulation of actin polymerization and depolymerization [23, 24]. Cholesterol oxides, liarozole, farnesol, and overexpression of Gas2 cause disruption of actin filaments and apoptosis, thereby showing a causal relationship between actin cytoskeleton reorganization or disorder and apoptosis [25–28]. We examined whether morphological changes in the actin cytoskeleton were associated with the reduced amount of thymosin β_4 in antitumor drug-induced apoptosis, and found that the actin filaments were reorganized into filament clumps (Fig. 6). When the effects of the drugs on the induction of apoptosis, amount of thymosin β_4, and organization of the actin fibers were compared, etoposide was the most effective drug, whereas ICRF-193 was the least. These results suggest that the decrease in thymosin β_4 alters the actin cytoskeleton organization and leads to apoptosis. Our observation is supported by findings that modulation of thymosin β_{10} expression affects the susceptibility of cells to apoptosis [29]. However, the involvement of the other actin binding proteins and actin itself in the reorganization of the cytoskeleton must also be considered. Although actin is reported to be cleaved in apoptotic cells, another paper has appeared showing that actin is resistant to cleavage *in vivo* [30].

To assess whether decreased thymosin β_4 is caused by the decreased expression of thymosin β_4 mRNA, we used RT-PCR. RT-PCR analysis demonstrated that the level of thymosin β_4 mRNA in antitumor drug-treated cells remained approximately the same as in non-treated control cells (data not shown). This suggests that the reduced level of thymosin β_4 was not caused by a decrease in its mRNA.

Caspases are activated sequentially in apoptosis, and they cleave enzymes involved in cell function and DNA repair, such as lamins, and poly(ADP-ribose) polymerase [7]. Thus, it is presumed that the reduced amount of thymosin β_4 is due to its cleavage by a caspase. Thymosin β_4 contains the DXXD cleavage motif for caspases, which is located at the

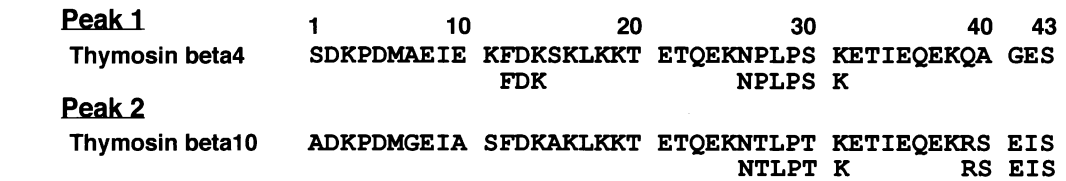

FIG. 5. Identification of intracellular proteins in Molt-4 cells. The peak numbers correspond to the peaks in Fig. 4.

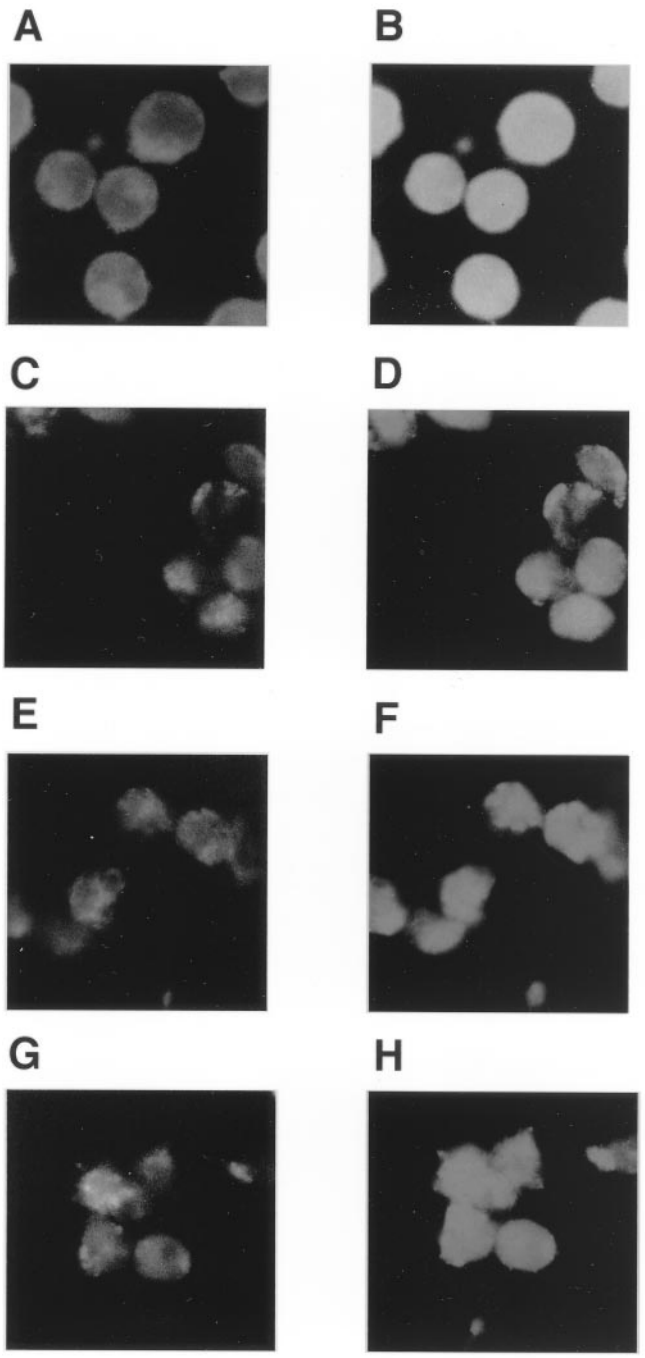

FIG. 6. Changes in the morphology of the actin fiber and localization of thymosin β_4 during apoptosis. Control cells and treated cells were stained simultaneously with rabbit anti-thymosin β_4 antibody and rhodamine-phalloidin. Left panels, phalloidin staining; right panels, thymosin β_4 staining. A and B: control cells. C and D: cells treated with 1 μM etoposide. E and F: cells treated with 20 μM ICRF-193. G and H: cells treated with 5 μg/mL of neocarzinostatin.

2–5 position from the N-terminal [31]. It is worth pointing out that AcSDKP, which is generated from thymosin β_4 digestion by endopeptidase, is an inhibitor of hematopoietic stem cell proliferation [32, 33]. It is intriguing that this cleavage sequence is also recognized by caspase 3. Thus, it is likely that the cleaved thymosin β_4 is involved in both the inhibition of cell growth and induction of apoptosis. We previously showed that zinc induces necrosis in LNCaP or PC-3 cells, followed by an increased amount of β-thymosins [34]. However, currently we have found that a variety of antitumor drugs did not affect the amount of β-thymosins in the necrotic cells. Here, we show that antitumor drugs induced apoptosis and reduced the amount of β-thymosin in Molt-4 cells. A reduced amount of thymosin β_4 is closely associated with morphological changes of the actin cytoskeleton in these drug-treated cells, suggesting a relationship between induction of apoptosis and reduced thymosin β_4.

If thymosin β_4 is indeed involved in the induction of apoptosis, the combination of antitumor drugs and modulation of thymosin β_4 expression could be a useful strategy for advanced chemotherapy.

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