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Potassium and Sodium Balance in U937 Cells During Apoptosis With and Without Cell Shrinkage

Valentina E. Yurinskaya, Tatyana S. Goryachaya, Irina V.Guzhova, Alexey V. Moshkov, Yuriy M. Rozanov, Galina A. Sakuta, Anna V. Shirokova, *Ekaterina V. Shumilina, Irina O. Vassilieva, *Florian Lang and Alexey A. Vereninov

Institute of Cytology, Russian Academy of Sciences, St. Petersburg; *Department of Physiology, University of Tübingen

Key Words

Apoptosis • Cell water • Cell sodium • Cell potassium • Etoposide • Staurosporine

Abstract

Staurosporine (STS) and etoposide (Eto) induced apoptosis of the human histiocytic lymphoma cells U937 were studied to determine the role of monovalent ions in apoptotic cell shrinkage. Cell shrinkage, defined as cell dehydration, was assayed by measurement of buoyant density of cells in continuous Percoll gradient. The K^+ and Na^+ content in cells of different density fractions was estimated by flame emission analysis. Apoptosis was evaluated by confocal microscopy and flow cytometry of acridine orange stained cells, by flow DNA cytometry and by effector caspase activity. Apoptosis of U937 cells induced by 1 μM STS for 4 h was found to be paralleled by an increase in buoyant density indicating cell shrinkage. An increase in density was accompanied by a decrease in K^+ content (from 1.1 to 0.78 mmol/g protein), which exceeded the increase in Na^+ content (from 0.30 to 0.34 mmol/g) and resulted in a significant decrease of the total K^+ and Na^+ content (from 1.4 to 1.1 mmol/g). In contrast to STS, 50 μM Eto for 4 h or 0.8-8 μM Eto for 18-24 h induced apoptosis without triggering cell shrinkage. During apoptosis of U937 cells induced by Eto the intracellular K^+/Na^+ ratio decreased like in the cells treated with STS, but the total K^+ and Na^+ content remained virtually the same due to a decrease in K^+ content being nearly the same as an increase in Na^+ content. Apoptotic cell dehydration correlated with the shift of the total cellular K^+ and Na^+ content. There was no statistically significant decrease in K^+ concentration per cell water during apoptosis induced by either Eto (by 13.5%) or STS (by 8%), whereas increase in Na^+ concentration per cell water was statistically significant (by 27% and 47%, respectively). The data show that apoptosis can occur without cell shrinkage-dehydration, that apoptosis with shrinkage is mostly due to a decrease in cellular K^+ content, and that this decrease is not accompanied by a significant decrease of K^+ concentration in cell water.

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A.A. Vereninov
Institute of Cytology, Russian Academy of Sciences
Tikhoretsky Av., 194064 St. Petersburg (Russia)
E-Mail veren@mail.cytspb.rssi.ru

Introduction

Apoptosis, or programmed cell death, is a fundamental biological mechanism to remove abundant, aged, defective, infected or potentially harmful cells [1-11]. It is generally believed that cell shrinkage is one of the essential characteristics of apoptosis [2, 3, 5, 6]. Ample evidence indicates that cell shrinkage during apoptosis is associated with loss of intracellular potassium [2, 3, 5, 6, 12-14].

The role of cell shrinkage in apoptosis remained, however, ill-defined. Apoptosis can be triggered by osmotic cell shrinkage, pointing to a causal role of cell shrinkage in the machinery eventually leading to cell death [15]. Moreover, apoptosis induced by other stimulators was shown to be paralleled by activation of K^+ and/or Cl^- channels [2, 3, 5, 6, 12-14, 16-18], inhibition of Na^+/H^+ exchange [19] and release of the osmolyte taurine [20], all effects favouring cell shrinkage.

However, apoptosis is not necessarily disrupted by absence of cell shrinkage [21]. Moreover, moderate osmotic cell shrinkage has even been shown to impede CD95 induced apoptosis in Jurkat T lymphocytes [22], challenging the view that cell shrinkage is necessarily triggering apoptosis.

Most recently we observed that etoposide (Eto) induced apoptosis of U937 cells without inducing cell shrinkage [23]. The goal of the present study was to compare the ion balance in U937 cells with and without apoptotic cell shrinkage. The data show that apoptosis can occur without cell shrinkage-dehydration, that apoptosis with shrinkage is mostly due to a decrease in cellular K^+ content, and that this decrease is not accompanied by significant decrease of K^+ concentration in cell water.

Materials and Methods

Cell culture

The human histiocytic lymphoma cell line U937 was obtained from the Russian Cell Culture Collection (Institute of Cytology, Russian Academy of Sciences, St. Petersburg, cell line U937 catalogue number $160B_2$). Cells at a concentration of $(0.7\text{-}1.0) \times 10^6$ cells/ml were incubated in RPMI at 37°C, and 5% CO_2. For induction of apoptosis, the cells were exposed to staurosporine (STS; Sigma-Aldrich, Steinheim, Germany) or etoposide (Eto; Sigma, Taufkirchen, Germany). Stock solutions of STS (2 mM) and Eto (34 mM) in DMSO were used to yield final concentrations of 1 µM STS and 50 µM Eto. The final concentrations of DMSO were 0.05% and 0.16%, respectively. There was no visible effect of the carrier alone.

Determination of cell water content

Cell water was determined by measurements of the buoyant density of cells in continuous Percoll gradient (Pharmacia, New Jersey, USA). The Percoll solutions were prepared by diluting the initial stock solution with RPMI medium to 40% and adding ×10 Hanks medium to maintain osmolarity. The density gradient was formed by centrifugation of the Percoll solution (2 ml) in 95 mm- length tubes for 40 min at 2000 *g* (K-23 centrifuge, Janetzki, Germany). Density marker beads of 1.033, 1.049, and 1.062 g/ml (Sigma, Taufkirchen, Germany) were used to control the gradient, whose steepness was about 0.005 g/ml/cm. The concentrated cell suspensions (100 µl, (3-5) $\times 10^6$ U937 cells) were placed on the Percoll solution surface and centrifuged for 10 min at 400 *g* (MPW-340 centrifuge, CHEMARGO, Blachownia/Czêstochowy, Poland). After isopycnic distribution of the cells in a density gradient, the fractions were collected with a pipette and placed into 1.5-ml Eppendorf tubes. Then they were diluted 4-6 times with RPMI medium and spun for 5 min at 300 *g* (MPW-310 centrifuge, CHEMARGO, Blachownia/Czêstochowy, Poland). The cells were resuspended in RPMI medium and used for ion determinations, microscopy, and flow cytometry.

The water content per g protein, v_{prot}, was calculated as $v_{prot.} = (1\text{-}\rho/\rho_{dry})/[0.65(\rho\text{-}1)]$, where ρ is the measured buoyant density of the cells and ρ_{dry} is the density of the cell dry mass, the latter taken as 1.45 g/ml. The ratio of protein to dry mass was taken as 0.65. The buoyant density of cells is a more sensitive and reliable measure for cell water content than all known techniques using intra- and extracellular water markers. A difference of buoyant density by 0.005 g/ml, which corresponds to a change in cell water content per g of cell protein by about 10%, yields a displacement of cells in gradient tubes by about 1 cm.

Determination of the cell ion content

The cells were pelleted and washed in $MgCl_2$ solution (96 mM) 5 times without resuspension. The pellets were treated with 1 ml of 5% trichloroacetic acid (TCA) for 30 min and TCA-extracts were analyzed for [K] and [Na] by emission photometry in an air-propane flame by using a Perkin-Elmer AA 306 spectrophotometer and solutions of KCl and NaCl (10-100 µM) in 5% TCA as standard. The TCA precipitates were dissolved in 0.1 N NaOH and analyzed for protein by the Lowry procedure with serum bovine albumin as standard. The cell ion content was calculated in mmol per g of protein.

Flow cytometry

Fluorescence of cells stained with acridine orange (AO) and ethidium bromide (EB) was determined using a flow cytometer equipped with a mercury arc lamp and a filter of 450-490 nm for fluorescence excitation [24]. For staining, the cells (1×10^6/ml) were incubated for 15-20 min at room temperature with AO (5 µg/ml) and EB (20 µg/ml). The fluorescence was measured at 530 ± 5 nm (F_{530}) and > 620 nm (F_{620}). For determination of the DNA content in cells and cell fragments the samples were placed into a solution containing 0.02% ethylenediaminetetracetic acid (EDTA), 15 mM $MgCl_2$, 0.1% Triton X-100, 20 µg/ml EB, and 40 µg/ml olivomycin,

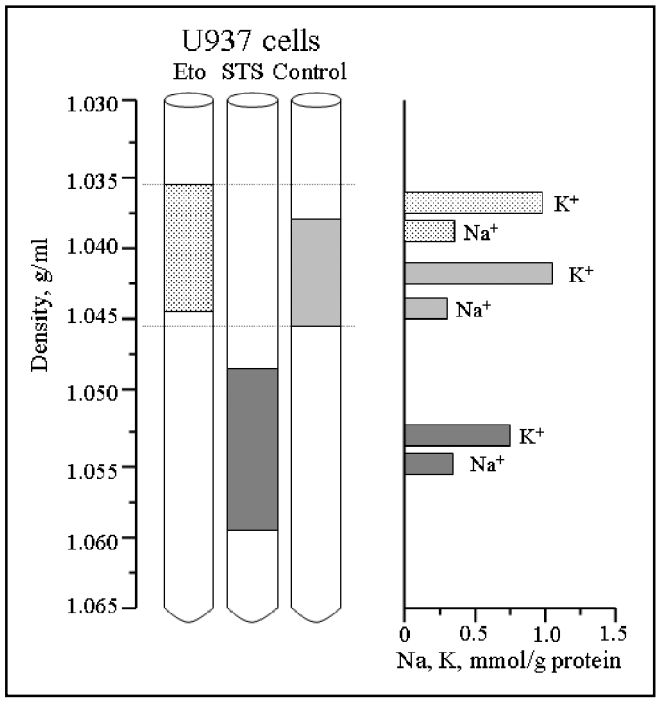

Fig. 1. Effects of etoposide- and STS-treatment on the distribution of U937 cells in continuous Percoll density gradient and on intracellular ion content. The U937 cells were treated with 50 μM Eto or 1 μM STS for 4 h. *Horizontal axis* – ion content, mmol/g protein. *Vertical axis* – density, g/ml. The ion content of cells and their position in tubes with density gradient are shown by a similar filling pattern.

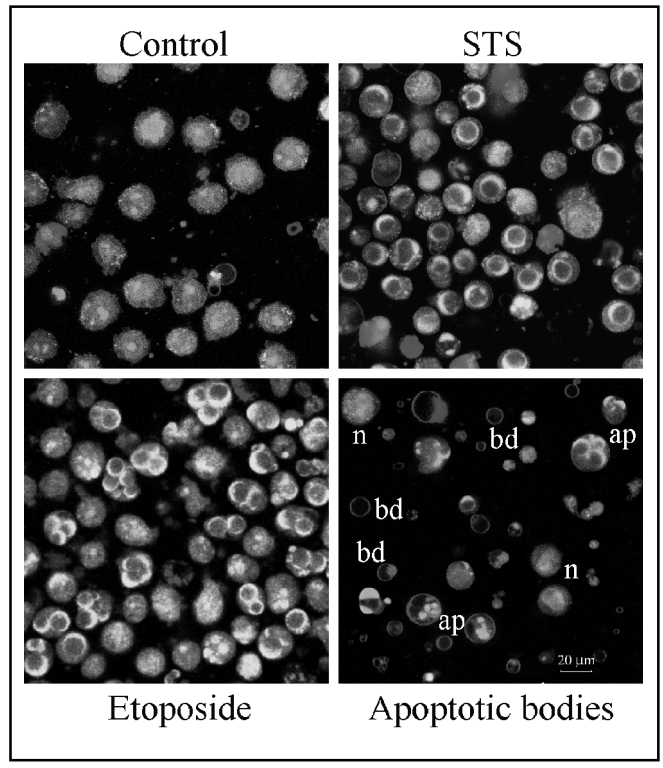

Fig. 2. Confocal images of U937 cells incubated for 4 h with STS (upper-right), Eto (bottom) or without inductors of apoptosis (upper-left). Cells incubated with 1 μM STS were subsequently stained with acridine orange (AO) and RH414. Cells incubated with 50 μM Eto were stained either with AO alone (left) or in a combination with RH414 (right, apoptotic bodies). A collection of apoptotic bodies was gathered from the different pictures. *n* – normal cells*; ap* – apoptotic cells*; bd* – apoptotic bodies.

pH = 7.4, for 20-24 h at 4-6 °C. Fluorescence was measured at > 600 nm and excitation within 380-470 nm using a flow cytometer [25].

Microscopy

Cell preparations were stained for 15 min with the lipophilic dye RH414 and (or) for 5 min with AO (5 μg/ml). In some experiments, cells were stained with AO in combination with EB (as for flow cytometry). The fluorescence was excited with an argon laser at 488 nm and passed through a 500-550 nm spectral window (for "green" fluorescence of AO) and 600-700 nm spectral window (for "red" fluorescence of AO, RH414, and EB fluorescence). A Leica TCS-SL microscope was used for confocal microscopy. Images were combined with a Zeiss LSM image browser.

Caspase activity

Caspase activity was determined using an *in vitro* test. In a typical experiment, U937 cells were taken 6 h after the Eto treatment to obtain lysates in the following solution: 25 mM HEPES, 1 mM EDTA, 5 mM ethyleneglycoltetracetic acid, 1 mM phenylmethylsulfonic acid, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. After centrifugation at 10000 *g* and measurement of protein concentration [26] the extracts (total protein 1 mg/ml) were placed into a 96-well plate and mixed with the reaction buffer (50 mM HEPES, 20% glycerol, 5 mM dithiotreitol) in triplets, to which the caspase-3/7 substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroanilidine (DEVD, Sigma, USA) was added to provide the final concentration of 100 μM. Dye release was measured using a Uniplan multiplate reader ("Picon" Incorporated Limited, Russia) at 405 nm.

Western Blotting

For immunoblotting of caspase activity, a polyclonal antibody against caspase-3 was used (generous gift of Dr. Yuri Lazebnik, Cold Spring Harbor Laboratory). Electrophoresis was performed with 13% polyacrylamide gels. After the electrophoretic transfer of protein bands onto nitrocellulose membranes [27] the membranes were incubated with the antibody against caspase-3 and subsequently with an

Cells	Buoyant density of cells, ρ, g / mL	Water, mL/g protein	K^+, mmol/g protein	Na^+, mmol/g protein	$[K^+]$, mM	$[Na^+]$, mM	K^+/Na^+	DNA cytometry data			
								S/G_1	G_2/G_1	Fr, %	n
Control	1.037 - 1.045 (7)	10.6	1.16±0.04	0.27±0.01	110	26	4.4±0.2 (20)	0.77±0.06	0.14±0.01	5.0±0.5	21
Eto	1.036 - 1.045 (7)	10.7	1.02±0.03	0.35±0.02[a]	95	33	3.0±0.2 (19)	0.32±0.02[a]	0.08±0.01[a]	26.5±3.8[a]	25
Control	1.040 - 1.046 (16)	10.0	1.1±0.03	0.30±0.02	110	30	3.9±0.2 (35)	0.74±0.06	0.13±0.01	5.8±0.9	18
STS 2 h	1.048 - 1.057 (8)	8.0	0.86±0.04	0.32±0.033	107	40	3.0±0.3 (14)	0.65±0.13	0.11±0.02	11.5±2.7	10
STS 4 h	1.049 - 1.060 (10)	7.7	0.78±0.03[a]	0.34±0.02[a]	101	44	2.7±0.2 (25)	0.76±0.10	0.19±0.03[b]	34.7±5.6[a]	14

Table 1. Water, K^+, and Na^+ content and ion concentration in apoptotic U937 cells. U937 cells were treated with 50 μM Eto for 4 h or 1 μM STS for 2-4 h. Water content per g protein, v, was calculated by formula: $v = (1-\rho/\rho_{dry})/[0.65(\rho-1)]$, using the density of cell dry mass, r_{dry}, taken as 1.45 g/mL and the share of protein in dry mass as 65 % . Data are the means ± SE. The number of experiments, n, is given in parentheses (for DNA cytometry – in the last column). a - the difference vs. appropriate control is significant at $P < 0.05$; b - the difference was insignificant at $P<0.05$ and significant at the $P = 0.08$ level with additional data from other experiments (n =35).

appropriate secondary antibody conjugated with horseradish peroxidase. The ECL-plus chemiluminescent cocktail (Amersham/ Pharmacia, UK) was used to develop the immunoblots.

Statistical analysis of results

The results were analyzed by Student's *t* test and considered statistically significantly different at $p < 0.05$.

Results

Apoptosis with and without cell dehydration

Fig. 1 illustrates schematically the distribution of U937 cells in a density gradient before and after induction of apoptosis by two different agents. Incubating the U937 cells with 1 μM STS for 4 h was followed by shifting the complete cell population towards high density. The density interval occupied by the apoptotic population was wider (0.011 ± 0.001 g/ml, $n = 10$, STS) than that for the control population (0.006 ± 0.001 g/ml, $n = 16$, control). A decrease in the cell water content resulted in an increase in cell density of the STS treated U937 cells amounting to about 2.3 ml/g protein (Table 1).

The density of the U937 cells did not appreciably increase after exposure of the cells to 50 μM Eto for 4-6 h. The apoptosis of U937 cells following prolonged exposure to 0.8-8 μM Eto for 18-24 h was also not followed by any cell shrinkage (1.039±0.0015 g/ml, n = 7 for Eto vs 1.040±0.0015 g/ml, n = 7 for control).

To explore whether STS and Eto, indeed, induced apoptosis, the cells were examined by flow cytometry and microscopy. Apoptosis of U937 cells treated with STS was confirmed by the typical "crescent" condensation of chromatin (Fig. 2) and by an increased percentage of nuclear fragments in DNA histograms (Fig. 3). Apoptosis of U937 cells treated with Eto was confirmed by the peculiar condensation of chromatin, formation of numerous apoptotic bodies, and a decreased portion of cells found in the S and G_2 phases in DNA histograms (Figs. 2 and 3). Western blotting and a DEVD-activity colorimetric assay showed that apoptosis in the Eto-treated U937 cells was accompanied by caspase-3 cleavage (Fig. 4). The data indicate that the STS- but not the Eto-induced apoptosis is paralleled by cell dehydration.

Intracellular K^+ and Na^+ during apoptosis with and without cell dehydration

As compared with intracellular K^+ content of control cells, the intracellular K^+ content per g cell protein was some 29% lower in shrunken STS-treated apoptotic cells, but only some 12% lower in unshrunken Eto-treated apoptotic cells (Table 1). The intracellular Na^+ content increased by 13% during the STS-induced apoptosis and by 30% during the Eto-induced apoptosis. The total K^+ and Na^+ content of STS-treated cells decreased significantly (by 20%), whereas in Eto-treated cells it remained virtually the same (-4%). Thus, apoptotic cell shrinkage correlated directly with a decrease in the total cellular K^+ and Na^+ content.

Our data allow to estimate quantitatively the relationship between a decrease in the total K^+ and Na^+ content and cell dehydration. Generally, the loss of cell

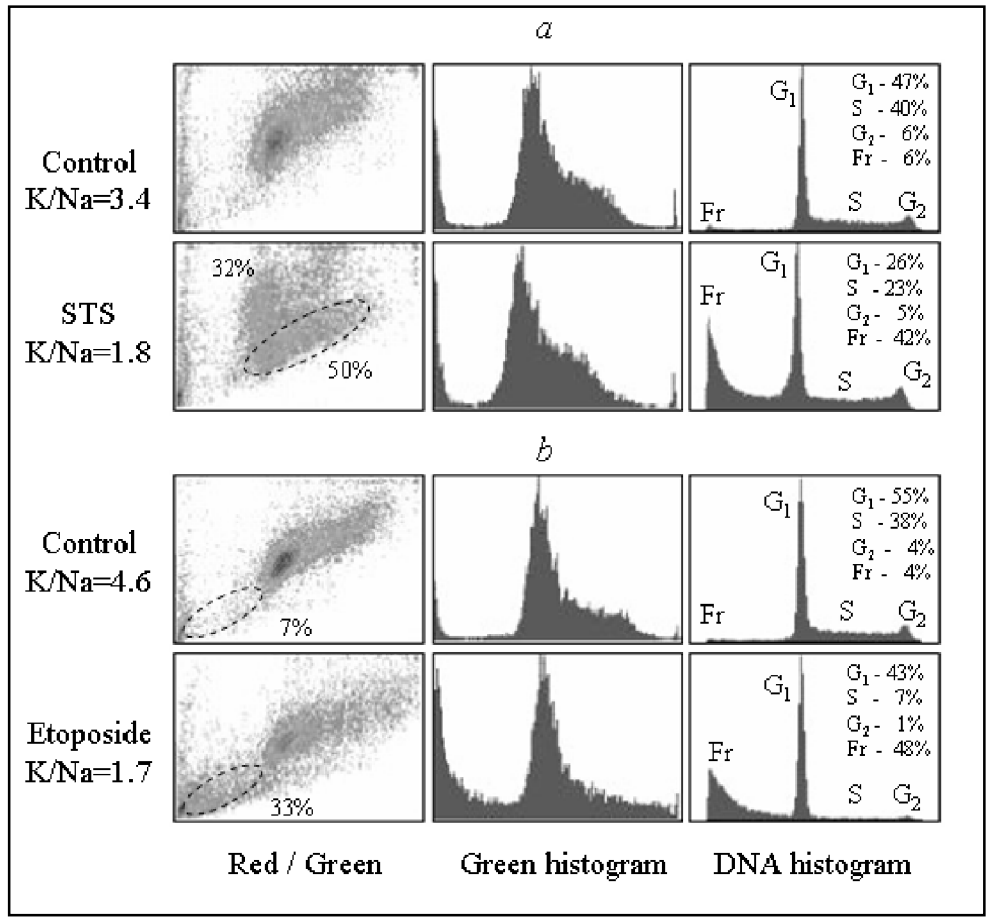

Fig. 3. Flow cytometry of U937 cells after incubation for 4 h with 1 μM STS (a) or 50 μM Eto (b). Data of two representative experiments with non-separated cells. *Horizontal axis*– fluorescence at 530 nm in two-dimensional Red/Green histograms and one-dimensional Green histograms, or the amount of DNA represented by BE fluorescence in DNA histograms. *Vertical axis* – fluorescence at 620 nm in two-dimensional Red/Green histograms or cell number in one-dimensional histograms. Symbols G_1, sG_1, S, G_2 and Fr designate cell subpopulations and nuclear fragments selected on the DNA histogram. Red /Green and Green histograms were obtained on cells stained with AO and EB. K/Na ratios for different variants of experiments are presented on the graphs.

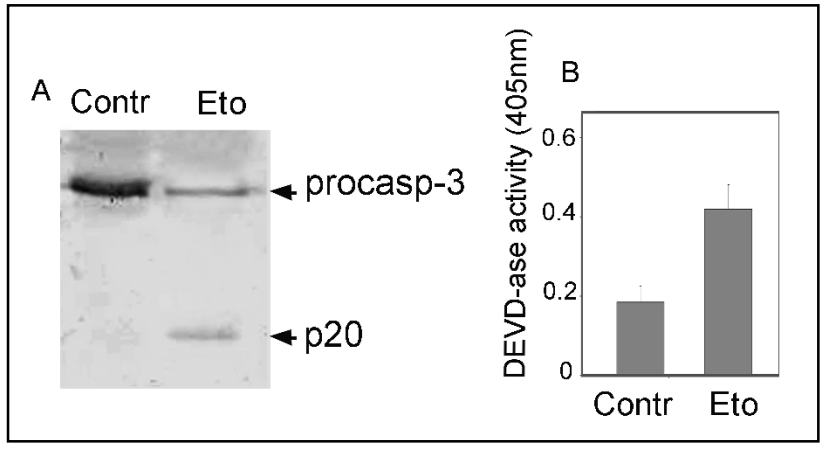

Fig. 4. Eto-induced apoptosis in U937 cells is accompanied by caspase-3 cleavage. Untreated U-937 cells and cells treated with 50 μM Eto for 6 h were divided into two parts. One part of cells was lysed in RIPA buffer and used for Western blotting with the use of anti-caspase3 antibody (A). The other part was lysed in "caspase-buffer" (see Material and Methods) and the lysate was incubated with DEVD-p-NA substrate. Provided are means ± SEM, n = 4 (B).

water (volume) under permanent osmotic equilibrium between cell and medium implies proportional isoosmolar exit of intracellular solutes and water. Hence, the decrease of water content of the STS-treated U937 cells by 2.3 ml/g protein yields a total osmolyte loss by 0.69 mmol/g protein, considering an osmolality of the external medium of 300 mOsm. The apoptotic decrease in the total K^+ and Na^+ content in these cells was found to be 0.28 mmol/g protein.

The concentrations of K^+ and Na^+ in cell water reported in Table 1 were calculated assuming a dry mass density of 1.45 g/ml. Using values of 1.4 or 1.5 g/ml yields K^+concentrations in control cells of 121 or 101 mM, respectively, instead of 110 mM. The Na^+ concentration

under the same conditions appears to be within the range of 28-24 mM. The K^+concentration during apoptosis of U937 cells induced by Eto and STS decreased only by 13.5 % and 8 %, respectively, an alteration not reaching statistical significance. Intracellular Na^+ concentrations during apoptosis of U937 cells induced by Eto and STS increased significantly by 27 and 47 %.

An additional series of experiments has been performed to explore whether Eto inhibits apoptotic cell shrinkage following treatment with STS. As a result, the mean buoyant density of cells was similarly high in cells treated with STS+Eto (1.050±0.002 g/ml, *n*=3) or STS alone (1.051±0.001 g/ml, *n*=2) and similarly low in cells treated with Eto alone (1.040±0.002 g/ml, *n*=3) or left untreated (1.041±0.001 g/ml, *n*=3). Thus, Eto did not prevent the decrease of cell volume during STS treatment.

Discussion

Apoptosis was defined historically as "shrinkage necrosis" [28]. It has been assumed until recently that cell shrinkage is an obligatory hallmark of apoptosis. Bortner & Cidlowski [21] were the first to show that apoptosis of anti-Fas treated Jurkat cells could occur without cell shrinkage. In their study, cell shrinkage was estimated indirectly by the forward light scattering in a flow cytometer. Moreover, separation of shrinkage from other characteristics of apoptosis was observed in cells incubated in sodium-substituted solutions. We demonstrate here another model of apoptosis without cell shrinkage-dehydration by using U937 cells treated with Eto i.e. a well established model of apoptosis [29, 30]. We believe that the paradigm that cell shrinkage is a necessary characteristic of apoptosis should be revised.

The absence of shrinkage during Eto-induced apoptosis in U937 cells is not a peculiar feature of Eto as an inducer. Eto induces apoptosis with shrinkage in rat thymocytes [31, 32]. There is no specific inhibitory effect of Eto on shrinkage of U937 cells, as apoptosis with shrinkage was observed when we incubated U937 cells simultaneously with STS and Eto. It was reported that STS and Eto initiated apoptosis via only partially overlapping pathways [33]. The difference in the final outcome of the apoptosis induced in U937 cells by Eto and by STS points to differences in the signaling of the two inducers of apoptosis. Moreover, there are further differences in the apoptotic phenotypes after treatment with STS and Eto. Numerous apoptotic bodies are formed during the Eto-induced, but not the STS-induced apoptosis. The percentage of cells in the S phase and the G_2/G_1 ratio decreased significantly in the Eto-treated cells (Table 1), whereas during the STS-induced apoptosis the S/G_1 ratio was not significantly changed, while the G_2/G_1 ratio increased. Similarly, the character of chromatin condensation in cells undergoing Eto-induced apoptosis differs from that observed in STS-induced apoptosis.

Apoptosis both with and without cell dehydration were accompanied by changes in the K^+ and Na^+ cell content and by a decrease in the K^+/Na^+ ratio. It is an evident and principal difference in changes of the total content of K^+ and Na^+, which leads to difference in cell shrinkage during compared types of apoptosis.

An estimate of the ionic share in the total apoptotic loss of osmolytes depends on the total osmolality of anions balancing a decrease in the total K^+ and Na^+ content of shrunken cells. It was shown by X-ray elemental microanalysis that the Cl and P content of apoptotic cells decreased in parallel with K [34]. If the accompanying anions are Cl^- and $H_2PO_4^-$, the total contribution of ions to the apoptotic exit of osmolytes from the STS-treated U937 cells yields 0.56 as compared to the total of 0.69 mmol/g protein, i.e. 81%. This is a maximal estimate, as a part of the K^+ exit may be paralleled by gain of H^+ leading to intracellular acidification [11, 19] and thus to a decrease of negative charge at intracellular proteins. Presumably more than 19% of the cell shrinkage are due to the exit of non-ionic osmolytes.

A decrease in the intracellular K^+ concentration is considered to play a pivotal role in regulation of apoptotic nucleases and caspases [35, 36]. We found that apoptosis of U937 cells treated with STS or Eto was associated with only a small, if any, decrease in K^+ concentration per cell water. A decrease in the cytosolic K^+ concentration during apoptosis of rat thymocytes treated with dexamethasone or Eto did not exceed one third [32]. The effect of K^+ on activity of these enzymes was shown in cell-free system under variation of K^+ concentration in a wide range. However, a decrease in the cytosolic K^+ concentration in thymocytes observed in vivo during apoptosis induced by dexamethasone or Eto did not exceed 35% [32]. Apoptosis of U937 cells treated with STS or Eto was associated with even less (8-13%) decline in concentration of K^+ in cell water. It should be mentioned that the change in concentration of K^+ acting as a cell volume regulator is always much less than K^+ content. The date obtained indicate that the exit of K^+ during apoptosis is significant mostly because of the volume

regulation but not of the modulation of activity of apoptotic enzymes. An increase in the intracellular Na^+ concentration during apoptosis in our experiments was pronounced by approaching +27% in the Eto-treated and +47% in the STS-treated U937 cells. Further studies are needed to reveal the role of this Na^+ increase in the stimulation of apoptosis.

In conclusion, distinct differences are observed in the water balance and parallel changes in the total intracellular K^+ and Na^+ content during the STS- and Eto-induced apoptosis of U937 cells. Apparently, neither substantial decreases of the cytosolic K^+ concentrations nor cell shrinkage are required for the induction of apoptosis in these cells.

Acknowledgements

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