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Regulatory Volume Increase (RVI) and Apoptotic Volume Decrease (AVD) in U937 Cells in Hypertonic Medium

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Abstract—Changes in intracellular water, K^+ and Na^+ of U937 cells incubated in hyperosmolar medium supplemented with 200 mM sucrose have been studied. Cells were stained with acrydine orange, ethydium bromide, APOPercentage dye, which marks the phosphatidyl serine distribution on the plasma membrane; and FLICA polycaspase fluorescent dye. It was found that cell shrinkage produced by direct osmotic effect induced both a regulatory volume increase and apoptotic volume decrease. The regulatory volume increase dominated at the early stage, whereas apoptotic volume decrease prevailed at the later stage. Therefore, U937 cells were capable of triggering apoptosis and apoptotic volume decrease, despite the unimpaired regulatory volume increase response, and the current opinion that the dysfunction of the regulatory volume increase is a prerequisite for apoptosis and apoptotic volume decrease (Subramanyam et al., 2010) should be revised. It is concluded that the apoptotic volume decrease plays a significant role in preventing osmotic lysis in apoptotic cells, rather than in initiating apoptosis.

Keywords: apoptosis, APOPercentage dye, FLICA, cell volume regulation, osmotic stress, buoyant cell density, U937 cells water, K^+, Na^+

Abbreviations used: APOP, APOPercentage dye; AO, acridine orange; EB, ethydium bromide; AVD, apoptotic volume decrease; RVI, regulatory volume increase; RVD, regulatory volume decrease

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The water imbalance and changes in volume in cells in hypo- or hypertonic medium trigger multiple cellular processes, known as "osmotic stress." Osmotic stress almost always is accompanied with "regulatory" changes in water balance and cell volume. Cell swelling in hypotonic media causes the reaction of regulatory volume decrease (RVD). Hypertonic media, that induce the loss of water and a reduction in the cell volume, cause the reaction of a regulatory volume increase (RVI). It is known that apoptosis whatever the cause it has been induced is accompanied by cell dehydration and reduction of cellular volume called named apoptotic volume decrease (AVD). The researchers who coined the term "AVD" believed that the accidental triggering off the RVD reaction under isotonic conditions plays an important role in apoptosis. (Maeno et al., 2000). Hence, AVD and RVD were considered to be analogous terms and the idea was put forth that apoptosis may be induced by osmotic cell shrinkage in hypertonic medium. In fact, the incubation of the cell in hypertonic medium induced apoptosis (Bortner and Cidlowski, 1996; Burg et al., 2007; Hoffmann et al., 2009). Currently, it is assumed that disorder in the cell volume regulation associated with apoptosis consists of impairing the RVI mechanism (Okada et al., 2001; Subramanyam et al., 2010). This assumption is widely supported.

This study describes the phenomenon of triggering both AVD and RVI in U937 cells cultivated in hypertonic medium. The induction of apoptosis in cells that retain the RVI reaction indicates that the widespread idea of the induction of apoptosis due to disorders in RVI (Subramanyam et al., 2010) should be revised. It is concluded that AVD is a phenomenon that underlies the prevention of osmotic lysis in apoptosis, rather than the initiation of apoptosis.

MATERIALS AND METHODS

Human histiocytic lymphoma U937 cells were obtained from the German Collection of Microorganisms and Cell Cultures. Cells were maintained in RPMI 1640 medium (Biolot, Russia) with 10% fetal calf serum (HyClone, United States) at 37°C and 5% CO_2. The experiments were performed on cells from one unfrozen batch cultured for 4–12 passages. Cells were plated with a density of $1.0–1.5 \times 10^6$ cells/ml. The hypertonic media were obtained by the addition of 200–300 mM sucrose. After 0.5, 2, and 4 h of incubation in hypertonic medium, the buoyant cell density was measured by the cell distribution in a Percoll gradient (Pharmacia, Sweden). A continuous Percoll gradient was prepared as described (Vereninov et al., 2004) with the addition of 200 or 300 mM sucrose.

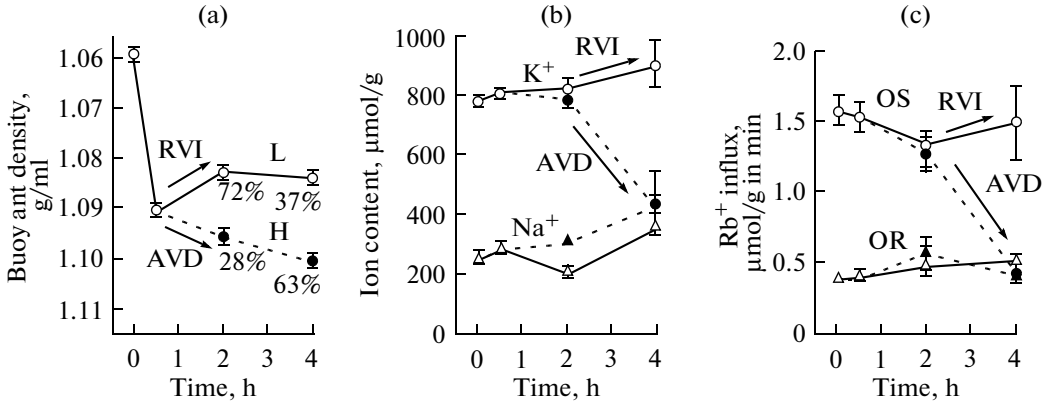

Fig. 1. (a) Buoyant cell density, (b) K^+ and Na^+ intracellular content, and (c) influx of Rb^+ influx through pump (OS) and ouabain-sensitive route (OR) in U937 cells incubated in RPMI 1640 medium supplemented with 200 mM sucrose. 0 h point corresponds to isotonic medium. Ion content is given in μmole per 1 g protein; Rb^+ influx is presented in μmole per 1 g protein per 1 min. Solid lines represent cells of initial and light subpopulations; dotted lines represent cells of heavy subpopulation. (a) % indicates number of cells in light (L) and heavy (H) fractions with corresponding average density. Arrows designate RVI or AVD response. Each point is mean value with standard deviation from three to five experiments.

The density of the density marker beads (DMB-10, Sigma-Aldrich, Germany) in sucrose containing Percoll medium was taken according to the manufacturer's recommendations and additionally checked by the gravimetric method. It was found that the changes in density in sucrose-saline media correspond to the manufacturer's diagrams for sucrose solutions. The Rb^+ influx was determined after the fractionation of the cell for 10 min in the buoyant density gradient by adding RbCl to the medium up to a final concentration of 2.5 mM. Rb^+ influx via the Na^+/K^+ pump was evaluated by the difference between the Rb^+ uptake in the presence and absence of 0.1 mM ouabain (Sigma, United States). The intracellular content of K^+, Na^+, and Rb^+ ions were assayed by flame emission spectrometry with Perkin-Elmer spectrophotometer as described (Yurinskaya et al., 2005). The protein content was determined by the Lowry method. The water content (v) per 1 mg protein was calculated according to the formula $v = (1 - \rho/\rho_{dry})/[0.79(\rho - 1)]$, where ρ is the cellular buoyant density, ρ_{dry} is the density of the dry cellular mass. The density of the dry cellular mass was considered to be equal to 1.35 g/ml and the protein content in the dry mass was regarded as 0.79.

Living cells stained with acridine orange (AO) and ethidium bromide (EB) (Serva, Germany) mixture or fluorescent labeled inhibitor of caspases (FLICA) apoptosis detection kit (ImmunoChemistry Technologies, United States) were observed under a Leica TCS SL confocal microscope with excitation lasers at 488 and 532 nm for AO/EB dyes and 488 nm for FLICA. Fluorescence was registered at 500–550 and 600–700 nm. Living cells stained with APOP (APOPercentage Dye, Biocolor, United Kingdom) were assayed under an Axiovert 200 M microscope equipped with a Leica DFC 420 digital camera. 5 μl dye (10 μg/ml AO and 20 μg/ml EB) or APOP were added to 100 μl cell suspension and incubated for 5 min with AO/EB or 30 min with APOP at 37°C. The presence of dye in the medium did not affect the microscopic assay of the cell. The cell was treated with FLICA according to the manufacturer's instructions, i.e., lyophilized FLICA was dissolved in 50 μl DMSO and diluted five times with the supplied buffer. 5 μl of this solution were added to 300 μml cell suspension and incubated for 1 h at 37°C. Cells were centrifuged, washed twice with the buffer, suspended in 10–20 μl, and analyzed under a microscope. The results were statistically analyzed using with Student's t test. The difference was considered to be significant at $p < 0.05$.

RESULTS

An increase in the osmolality of the sucrose-containing medium resulted in a rapid increase in the buoyant density of the cell due to the loss of intracellular water. The water content of these cells calculated from the buoyant density was initially 43% lower than in the control cells. Two cell subpopulations with different buoyant densities appeared after fairly long incubation (2–4 h). Figure 1a demonstrates the changes in the average cell buoyant density and the quantity of cells estimated by the total protein content in each subpopulation. The water content in cells of the light subpopulation increased by 12% in interval 0.5–2, then did not change 3. In cells of the heavy subpopulation, the water content decreased by 14% after 4 h of incubation compared to cells incubated for 0.5 h. Both the buoyant density of the cells and the cell distribution between fractions changed after incubation in the hypertonic medium. About 28% cells were found in the heavy subpopulation after incubation in medium with 200 mM sucrose for 2 h; their number increased to 63% after 4 h incubation. A similar pat-

tern of cell distribution was observed with cells incubated with 300 mM sucrose. The results show that hypertonic medium triggers two reactions, i.e., fast RVI and slow water loss, which resemble AVD.

The transition of a cell from a light to heavy (apoptotic) subpopulation is accompanied by diminished intracellular K^+ content and augmented Na^+ content (Fig. 1b). The total amount of these ions was reduced, that is in good agreement with the decreased water content in these cells. The K^+/Na^+ ratio in cells of this subpopulation dropped drastically as was observed with U937 induced to apoptosis by staurosporine and etoposide (Yurinskaya et al., 2005).

Figure 1c illustrates an influx of Rb^+ in cells of light and heavy subpopulations incubated in hypertonic medium. In cells of the heavy population, a substantial decline was observed in ouabain-sensitive Rb^+ influx that mirrored the reduced K^+ transport through the Na^+/K^+ pump. We reported previously that this was a common feature for U937 apoptotic cells (Vereninov et al., 2007, 2008; Yurinskaya et al., 2010, 2011). Unlike cells with increased density, no drastic drop in the Rb^+ pump influx was observed in cells of the light population. The influx of ouabain-resistant Rb^+ was almost constant in both cell fractions independent of the time of incubation.

Microscopic assay shows that morphological changes are only visible in cells of heavy subpopulations. Figures 2 and 3 demonstrate that morphological modifications are barely noticeable in cells incubated with 200 mM sucrose for 1 h (Fig. 2b) or with 300 mM sucrose for 15 min (Fig. 3b). Under these conditions, the cell density is homogeneous in the population. Unlike the cells from the light population, cells of the heavy population usually lost their round shape (Figs. 2c, 2e). Figure 3d shows cells from an unfractionated population. Many cells were stained with apoptotic marker APOP after 2 h incubation with 300 mM sucrose. Currently, APOP is commonly used as a diagnostic marker for apoptosis. The dye staining is determined by lipid flip-flop transitions of plasma membrane lipids changed in apoptosis, which makes it possible for APOP to penetrate the cell. It is considered that APOP marks changes in the plasma membrane similar to those marked by annexin *V* (Keter et al., 2008). It should be noted that, occasionally, cells with dramatically altered morphology were not stained with APOP (Figs. 3e, 3f).

The number of cells stained with ethidium bromide was not increased in cultures with 200 or 300 mM sucrose. Thus, in our opinion, the changes in the ion composition and the influx of Rb^- in cells from the heavy subpopulation associated with AVD and apoptosis reflect relatively early stages of apoptosis. We have previously found that, in staurosporine- and etoposide-induced apoptotic U937 cells, the pattern of chromatin condensation revealed by AO is specific to a particular inducer (Vereninov et al., 2004; Yurinskaya et al., 2005). In cells incubated with 200 mM sucrose for 1 h (population is relatively homogeneous in cell density), only small translocations of AO-stained orange fragments from the periphery into the center of the cell may be visible. In these cells, chromatin staining with green AO was not changed compared to the control cells. After incubation with 200 mM sucrose for 4 h (Figs. 2c, 2e), cells of the heavy population could be distinguished in their morphology and AO staining from both control cells and cells from the light subpopulation (Figs. 2a, 2d). Some cells acquired a spindle-like shape (Figs. 2c, 2e). The small EB-stained fragments in the cells of the heavy fraction are obviously apoptotic bodies. Cells from the light fraction were very similar to the control cells. Thus, the pattern of AO/EB staining of heavy-population cells observed in cultures after prolonged cultivation with 200 or 300 mM sucrose was comparable to that visible in staurosporine- or etoposide-induced apoptotic U937 cells.

Living cells were tested with polycaspase fluorescent inhibitor FLICA. The dye irreversibly binds with activated caspases and, therefore, should stain the cells with active caspases into the green color. In black-and-white images, cells stained with FLICA should be white (the white arrows in Fig. 4 show a few cells stained with FLICA). Black cells are cells with no penetrated FLICA. In our experiments, the number of cells stained with FLICA after incubation with 200 mM sucrose for 4 h did not notably differ from control cultures (Fig. 4) although more than half of the treated cells displayed AVD (Fig. 1a). A possible reason for this is the delayed caspase activation compared to AVD (Subramanyam et al., 2010).

DISCUSSION

Many papers have been devoted to the search for mechanisms of cell volume regularion recovery in anisotonic medium (see reviews Hoffmann and Simonsen, 1989; O'Neill, 1999; Wehner et al., 2003; Okada, 2004; Hoffmann et al., 2009). The RVI response to osmotic pressure is observed in not all cells; therefore, it is usually studied on cells initially incubated in hypotonic medium, then transferred into medium with normal tonicity (RVI-after-RVD protocol). The reduced density of U937 after the cell was transferred from normal isotonic medium into hypertonic medium indicates that these cells, unlike other lymphoid cells currently examined, are able to the direct RVI response. This capacity made it possible to observe both the RVI and delayed AVD response in these cells.

RVI mechanisms were studied before the mechanisms of AVD; the abbreviation "AVD" has been used over the past 10 years (Bortner and Cidlowski, 1998, 2004, 2007; Lang et al., 1998, 2007; Maeno et al., 2000; Okada et al., 2001; Hoffmann et al., 2009). It is generally accepted that AVD is exhibited before many other hallmarks of apoptosis, such as caspase activa-

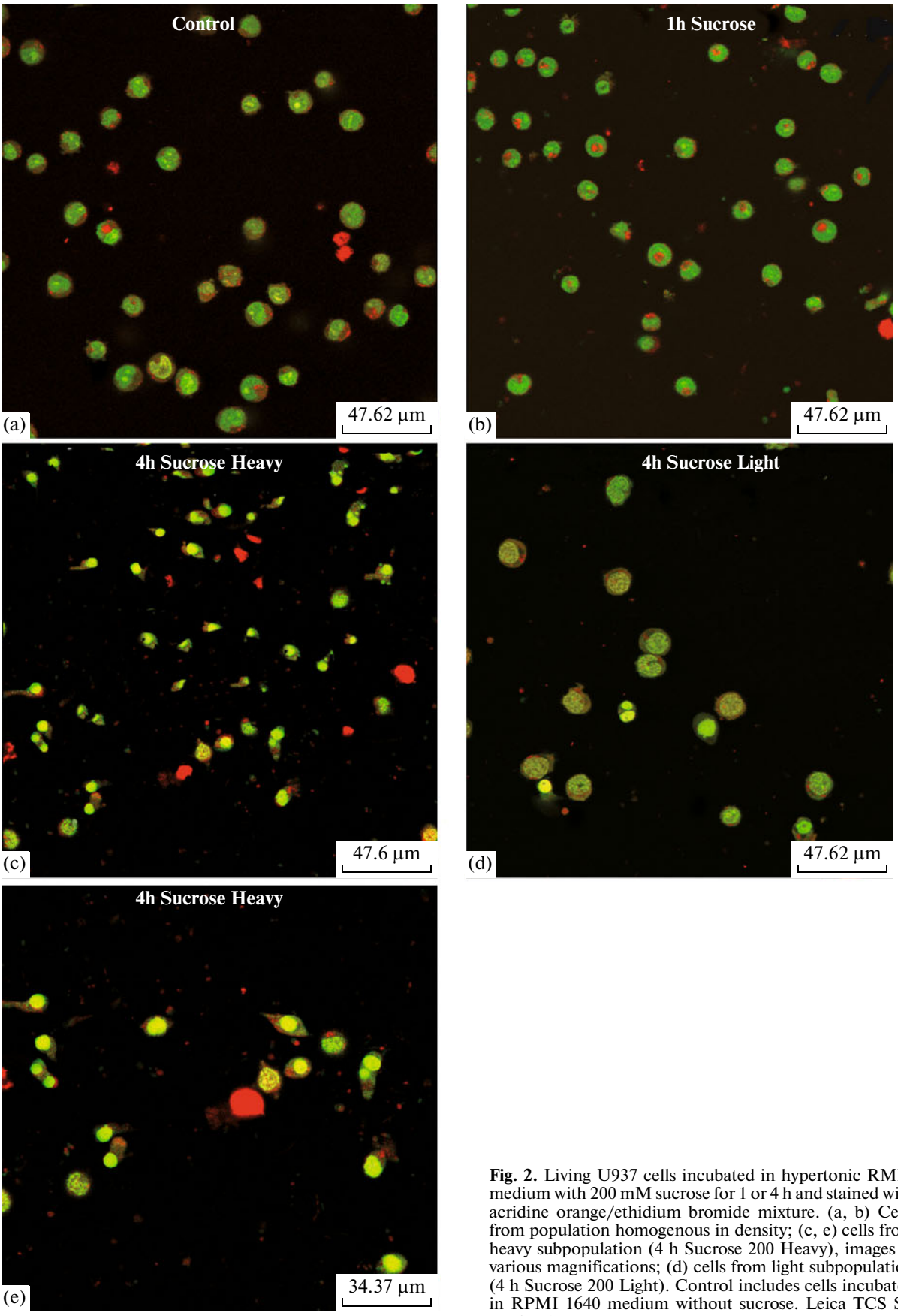

Fig. 2. Living U937 cells incubated in hypertonic RMPI medium with 200 mM sucrose for 1 or 4 h and stained with acridine orange/ethidium bromide mixture. (a, b) Cells from population homogenous in density; (c, e) cells from heavy subpopulation (4 h Sucrose 200 Heavy), images at various magnifications; (d) cells from light subpopulation (4 h Sucrose 200 Light). Control includes cells incubated in RPMI 1640 medium without sucrose. Leica TCS SL confocal microscope.

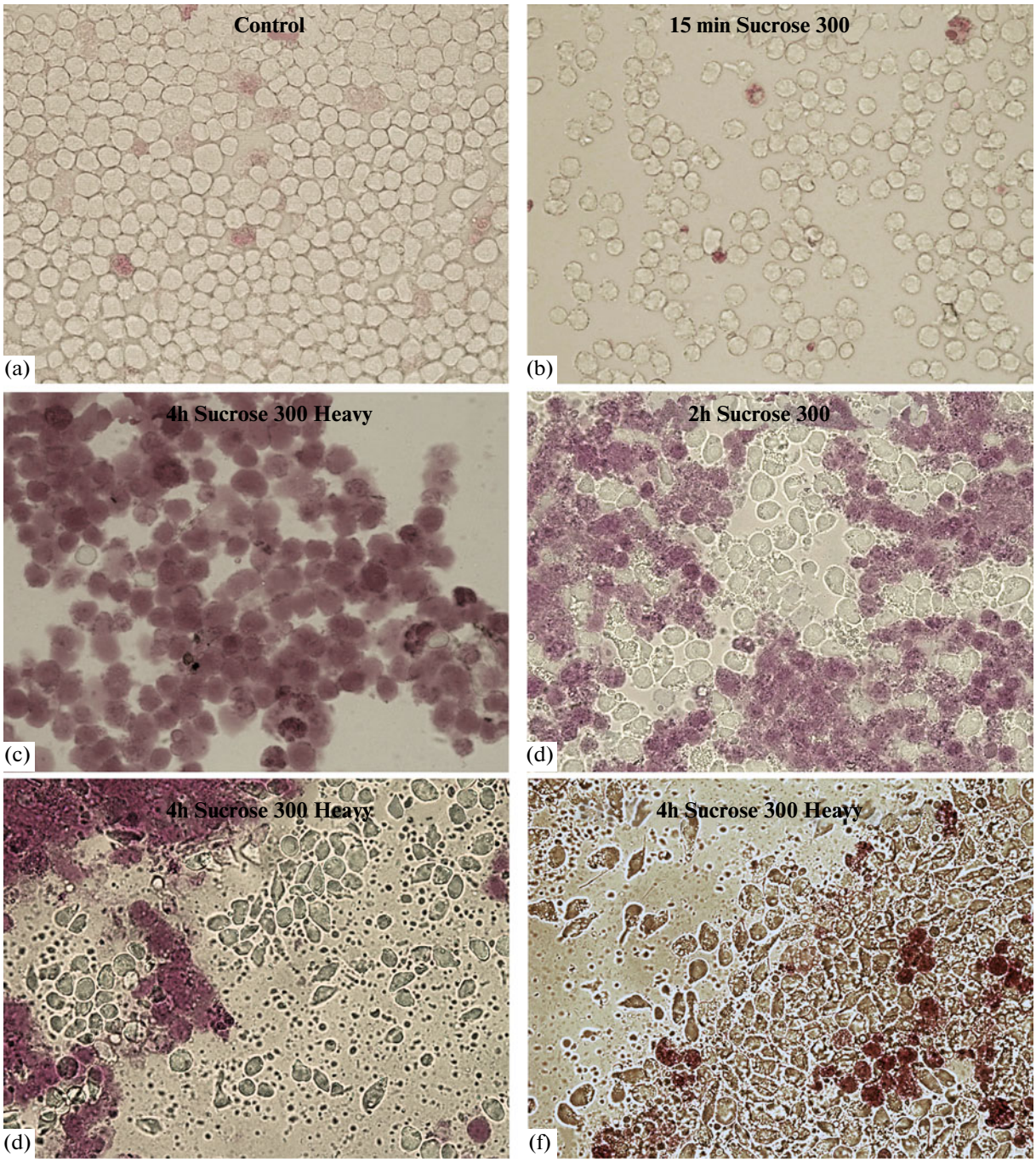

Fig. 3. Living U937 cells incubated in hypertonic RMPI 1640 medium with 300 mM sucrose for 15 min, 2 or 4 h and stained with APOP. (a, b) Cells from population homogenous in density; (c, e, f) cells from heavy subpopulation (4 h Sucrose 300 Heavy); (d) cells from unfractionated population (2 h Sucrose 300); (a, b, c), (d, f) and (e) results were obtained in three different experiments. Control includes cells incubated in RPMI 1640 medium without sucrose. Arrows indicate APOP-positive cells. Axiovert 200 M microscope equipped with Leica DFC 420 digital camera, obj. Plan-Neofluar 40×/0.75.

tion, the release of cytochrome c from mitochondria, DNA fragmentation, and chromatin condensation. Mouse T-lymphocyte shrinkage developed minutes after the induction of apoptosis was observed and before the phosphatidylserine translocation in the plasma membrane and annexin *V* binding were detected (Elliott and Higgins, 2003).

Microscopic observations of cells transferred into hypertonic medium showed that changes in the cell volume and the intracellular content of K^+, Na^+, and

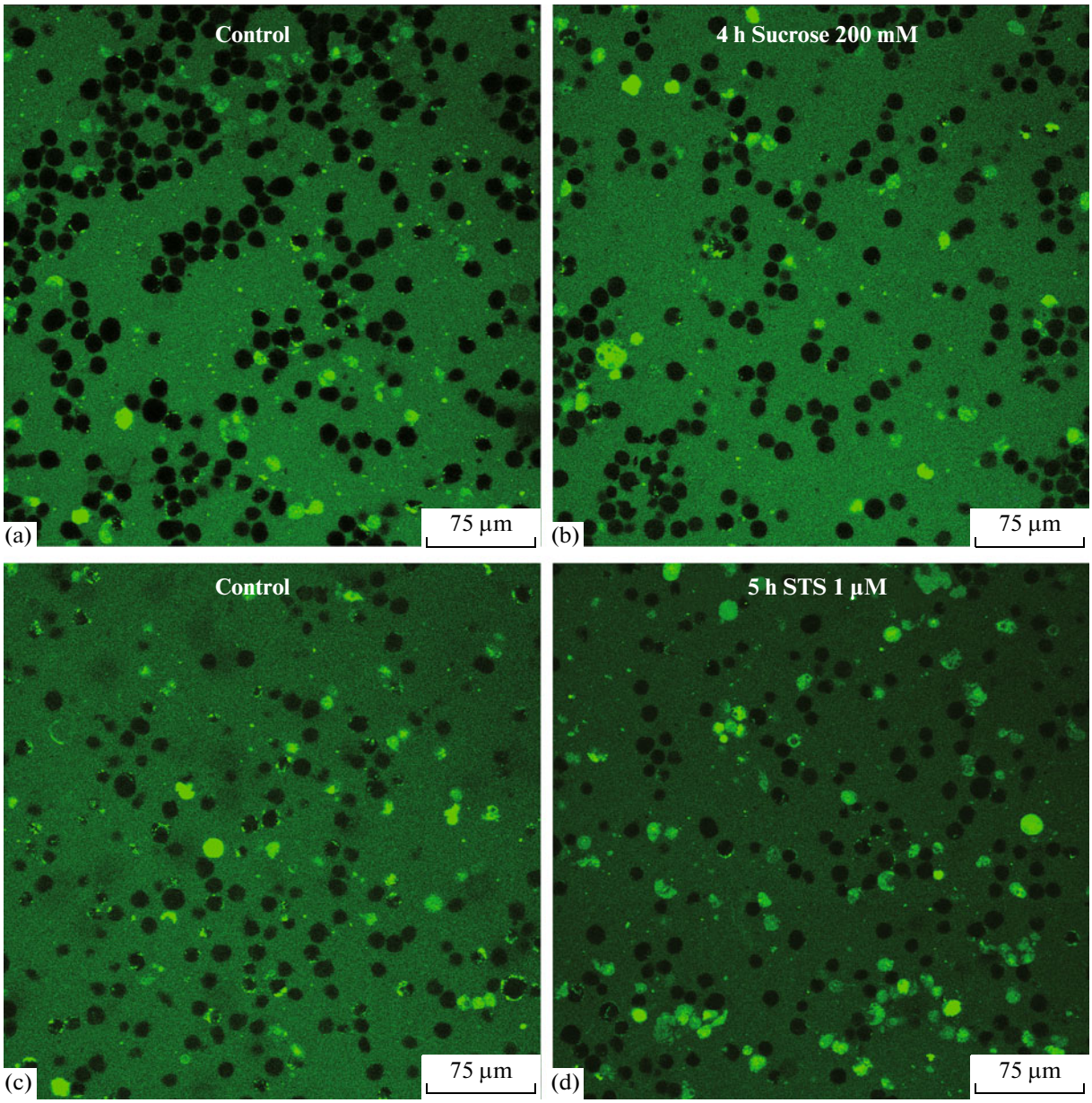

Fig. 4. Living U937 cells stained with FLICA poly caspase fluorescent dye. (a) Cells incubated in normal RPMI 1640 medium; (b) cells incubated in hypertonic RPMI 1640 medium with 200 mM sucrose for 4 h. White arrows show FLICA-positive cells. Leica TCS SL confocal microscope.

the influx of Rb^+ through the Na^+/K^+ pump considered in this paper are features of AVD and advanced polycaspase FLICA cell staining. The shapes of modified cells and apoptotic bodies, as well as AO and APOP staining, implied that the increased number of cells in the heavy subpopulation was due to apoptosis.

The induction of apoptosis by hypertonic cell shrinkage has been described in many papers (Bortner and Cidlowski, 1996; Edwards et al., 1998; Michea et al., 2000; Terada et al., 2001; Lang et al., 2002; Friis et al., 2005; Ghosh et al., 2007; Racz et al., 2007; Ernest et al., 2008). However, there are a few studies on concurrent RVI and AVD responses. The RVI response was observed in HL-60 cells placed into hypertonic medium with 300 mM mannitol. However, after 8 h, the cell volume was lower than the initial volume, i.e., AVD was observed. The number of apoptotic cells stained with annexin, but not propidium iodide increased in these cells in 8 h (Ghosh et al., 2007). Like our results, these data may be considered to support the exchange of RVI for AVD. Changes in the cell volume in apoptosis were studied on human glioblastoma D54-MG cells stimulated to apoptosis with various inducers, including hypertonic medium with

300 mM mannitol (Ernest et al., 2008). After the rapid decrease in the cell volume produced by the direct osmotic effect slows, a subsequent reduction in volume was registered, which, based on some features, the authors determined to be AVD. No well-defined RVI response was visible, but the addition of 2 mM glutamine inhibited the AVD response. The authors believe that the AVD response was compensated for by RVI because glutamine supplemented into hypertonic medium with lower osmolyte concentration (150 mM mannitol) produced obvious RVI (Ernest and Sontheimer, 2007). Taking into consideration the data mentioned above, the slow reducing volume of Ehrlich ascites cells in hypertonic medium described rather long ago (Hendil and Hoffmann, 1974; Levinson, 1991) is currently considered to be the AVD response.

The relationship between AVD, RVI, and RVD has been studied in detail by Japanese workers (Maeno et al., 2000; Okada et al., 2001; Maeno et al., 2006; Shimizu et al., 2006, 2007; Numata et al., 2008; Subramanyam et al., 2010). The interference between opposite AVD and RVI response was examined in HeLa cells (Numata et al., 2008; Subramanyam et al., 2010). The cell volume was assayed with the combined action of hypertony and traditional apoptosis inducers (staurosporine, TNFα and FasL). The following scheme was proposed. A hypertonic medium signal is transmitted via PI-3K kinase and a signal from isosmoticapoptosis inducers is transmitted via ASK1 kinase converge on serine/threonine kinase Akt1. If isosmotic apoptosis inducers are silent, the hypertonic medium signal activates Akt1 and causes normal RVI. Signals from isosmotic apoptosis inducers prevent the activation of Akt1 by hypertonic stress and exclude RVI. Thus, AVD is associated with an impairment of RVI (Subramanyam et al., 2010). The scheme is based on a large body of experimental data performed on HeLa cells; detailed studies of other cells have not been carried out.

This scheme (Subramanyam et al., 2010) is insufficient to explain the results we obtained with U937 cells because it does not consider time-dependent events. The authors of the suggested scheme did not observe the substitution of RVI for AVD in HeLa cells that we detected in U937 cells. Therefore, they concluded that the cell's inability to accomplish RVI resulted in apoptosis. Our experiments showed that apoptosis was developed in cells capable of the RVI response. Only later was RVI replaced with AVD. This implies that RVI is not an obstacle in early stages of apoptosis and, consequently, it is not the lack of RVI that triggers apoptosis with AVD observed at a certain stage. We believe that AVD underlies processes that prevent the osmotic swelling of the cell, rather than initiate apoptosis. It should be noted that it is currently stated that the development of apoptosis may be uncoupled with AVD (Bortner and Cidlowski, 2003; Vereninov et al., 2004; Yurinskaya et al., 2005; Hoffmann et al., 2009).

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