Original Paper

Dual Response of Human Leukemia U937 Cells to Hypertonic Shrinkage: Initial Regulatory Volume Increase (RVI) and Delayed Apoptotic Volume Decrease (AVD)

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Key Words

Apoptosis • Cell volume regulation • Osmotic stress

Abstract

Background/Aims: Osmotic cell shrinkage is a powerful trigger of suicidal cell death or apoptosis, which is paralleled and enforced by apoptotic volume decrease (AVD). Cells counteract cell shrinkage by volume regulatory increase (RVI). The present study explored the response of human U937 cells to hypertonic solution thus elucidating the relationship between RVI and AVD. **Methods:** Cell water, concentration of monovalent ions and the appearance of apoptotic markers were followed for 0.5–4 h after the cells were transferred to a hypertonic medium. Intracellular water, K⁺, Na⁺, and Cl[−] content, ouabain-sensitive and -resistant Rb⁺ influxes were determined by measurement of the cell buoyant density in Percoll density gradient, flame emission analysis and ³⁶Cl[−] assay, respectively. Fluorescent microscopy of live cells stained by acridine orange and ethidium bromide was used to verify apoptosis. **Results:** After 2–4 h incubation in hypertonic media the cell population was split into light (L) and heavy (H) fractions. According to microscopy and analysis of monovalent ions the majority of cells in the L population were healthy, while the H fractions were enriched with apoptotic cells. The density of L cells was decreasing with time, while the density of H cells was increasing, thus reflecting the opposite effects of RVI and AVD. At the same time, some of the cells were shifting from L to H fractions, indicating that apoptosis was gradually extending to cells that were previously displaying normal RVI. **Conclusion:** The findings suggest that apoptosis can develop in cells capable of RVI.

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Introduction

In a wide variety of cell types, hypertonic cell shrinkage results in apoptosis [1-18]. A distinctive feature of apoptosis is the apoptotic volume decrease (AVD), i.e. a decrease in cell volume generated by an intrinsic apoptotic machinery and associated with specific changes in the monovalent ion fluxes and content [1, 7, 19-26]. On the other hand, hypertonic shrinkage triggers regulatory volume increase (RVI), a common response of cells to water balance disturbance [12, 27]. The relationship between AVD and RVI provides additional insights into the physiology of apoptosis. It has been hypothesized that impairment of cell volume regulation plays a significant role in initiating apoptosis and AVD [20, 25]. We have previously reported that hypertonic shrinkage of human leukemic cells U937 initially caused RVI that was later superseded by the AVD response [28]. Accordingly, AVD and apoptosis may be triggered in cells which are still capable of RVI. The present study explored RVI and AVD in U937 cells in response to hypertonic shrinkage in greater detail. The question of how the same initial perturbation causes physically opposite physiological responses, is of importance beyond its relevance to apoptosis. U937 cells treated by hypertonic solution may thus be a useful model to study the switching of intracellular volume regulatory mechanisms from RVI to AVD.

Materials and Methods

Reagents

RPMI 1640 medium and fetal bovine serum (FBS, HyClone Standard) were purchased from Biolog (Saint Petersburg, Russia). Ouabain, 5-(N,N-dimethyl)-amiloride (DMA), 4,4-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS) were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions of DMA (10 mM in DMSO), ouabain (1 mM in phosphate salt buffer solution PBS) and DIDS (20 mM in water) were stored at 4 °C. Percoll was from Pharmacia (Uppsala, Sweden). Acridine orange (AO) and ethidium bromide (EB) were from Serva (Heidelberg, Germany). The isotope $^{36}\text{Cl}^-$ was from "Isotope" (Saint Petersburg, Russia). Sucrose of analytical grade was from Reagent (Saint Petersburg, Russia).

Cell cultures and media

Human histiocytic lymphoma U937 cells from the German Collection of Microorganisms and Cell Cultures (DSMZ) were maintained in RPMI 1640 medium with 10% fetal bovine serum 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 at a density of $(1-1.5) \times 10^6$ cells per ml. The hypertonic media were prepared by addition of 100 mM NaCl or 150-300 mM sucrose to the isotonic RPMI medium. All the incubations were done at 37 °C.

Determination of cell water content and cell volume

Cell water was determined by measurements of the buoyant density of cells in continuous Percoll gradient as described earlier [29, 30]. The osmolarity of Percoll solution was changed by adding 100 mM NaCl or 150-300 mM sucrose and checked with the Advanced Instruments Micro-osmometer Model 3320. The buoyant density of cells was estimated using density marker beads (DMB, Sigma-Aldrich, Germany). For the values of density of DMB in sucrose-containing Percoll medium manufacturer's data additionally confirmed by gravimetric measurements were used. After the cells achieved isopycnic distribution in a density gradient, fractions were collected, washed with RPMI and assayed for ion content. The buoyant density of cells proved to be a more sensitive and reliable measure of cell water content than techniques based on comparison of intra- and extracellular water marker distribution. A difference of buoyant density by 0.005 g ml^{-1} , which corresponds to a 10% change in cell water content per g of cell protein, yields a displacement of cells in gradient tubes by about 1 cm. The water content per g of protein, v_{prot} , was calculated as $v_{\text{prot}} = (1 - \rho/\rho_{\text{dry}})/[0.79(\rho - 1)]$, where ρ is the measured buoyant density of the cells and ρ_{dry} is the cell dry mass density; the latter was estimated as 1.35 g ml^{-1} [30]. Cell volume per g of protein was calculated as $\text{vol}_{\text{prot}} = (1 - \rho/\rho_{\text{dry}})/[0.79(\rho - 1)] + 1/0.79\rho_{\text{dry}}$.

Determination of intracellular ion content and Rb⁺ influx

Intracellular content of K⁺, Na⁺ and Rb⁺ ions was assayed by flame emission spectrometry with a Perkin-Elmer AA 306 spectrophotometer, as described previously [29]. Rb⁺ influx was determined after cell fractionation in the density gradient by adding 2.5 mM RbCl to the medium for 10 min. Rb⁺ influx via Na⁺/K⁺ pump was evaluated from the difference between Rb⁺ uptake in the presence and absence of 0.1 mM ouabain. To determine intracellular Cl⁻, cells were cultured for ≥90 min at 37°C in RPMI medium containing ³⁶Cl⁻ (0.12 μCi ml⁻¹). The radioactivity of ³⁶Cl⁻ was measured using a liquid scintillation counter (Beckman LS 6500), and the intracellular Cl⁻ content was calculated based on the specific activity of ³⁶Cl⁻ (~2 counts min⁻¹ μmol⁻¹). Cell protein was analyzed by the Lowry method with serum bovine albumin as a standard.

Microscopy

Living cells stained with acridine orange (AO, 10 μg/ml) and ethidium bromide (EB, 20 μg/ml) for 15-20 min were observed under a Leica TCS SL confocal microscope with excitation lasers 488 and 532 nm. Fluorescence was registered at 500-550 and 600-700 nm. Transmission images of cells in culture plates were obtained on an Axiovert 200M microscope equipped with a digital camera Leica DFC 420. Image analysis was performed using ImageJ 1.43(<http://rsbweb.nih.gov/ij/>).

Statistical analysis

The results were statistically analyzed using Student's t-test. Differences were considered to be significant at $p < 0.05$.

Results*Cell buoyant density, cell water*

Exposure of cells to hypertonic medium was followed by a prompt increase in cell buoyant density due to passive osmotic loss of cell water. The observed changes in cell density corresponded to a loss of cell water by 38-56% (depending on the osmolarity of the medium), i.e. to a decrease in the cell volume by 32-47% (Fig. 1A-D). Cells that shrank in hypertonic media initially formed a single band in the Percoll gradient that occupied a density interval of about 0.012 mg ml⁻¹. Following 2-3 h incubation, the cell population appeared to split into two subpopulations separated by a clearly detectable gap. Cell density in the light subpopulation (L) decreased relative to the density at time point 0.5 h, whereas the density of cells in the heavy subpopulation (H) slowly increased.

The ratio of H to L cell numbers (estimated by protein) increased over time, and by 4 h H cells comprised 50-60% of the total population (Fig. 1L, M). Accordingly, cells initially responded by RVI, but later started to shrink and shifted to the H fraction; this transition appeared to happen rather fast. The cell volume increase following the initial hypertonic cell shrinkage should be interpreted as an RVI. About 80-90% of U937 cells were able to increase their cell water content by 10.8-17.4% (corresponding to an 8.5-13.2 % volume increase) within 2 h relative to its lowest value at 0.5 h (without reaching the initial value in normotonic medium). Different cell types vary in their ability to restore their volume in hypertonic solutions. In some cells, RVI response is only observed when cells preincubated in hypotonic medium are returned to isotonic solution [27, 31]. Some cells are able to restore their volume more effectively than U937 cells [11, 32, 33]. Nevertheless, the present data reveal the ability of U937 cells to mount a partial RVI in response to direct transition from normotonic to hypertonic media.

Monovalent ions

The increase in cell hydration responsible for RVI was associated with an increase in cell K⁺ and Cl⁻ content (Fig. 1E-H, Fig. 2, Table 1). In turn, a decrease in cell water in H subpopulation was associated with a decrease in cell K⁺ and an increase in Na⁺. A decrease in Cl⁻ content was observed in the unfractionated cell population 4 h following hypertonic exposure, when the proportion of heavy cells was significant (Fig. 2). A decrease in Cl⁻ and

Fig. 1. Effect of hypertonic media on cellular characteristics. (A-D) Cell buoyant density and cell water content. Solid lines indicate unfractionated cells or light (K) cell subpopulation; dotted lines, heavy (H) cell subpopulation. (E-H) Intracellular K⁺ and Na⁺ content. (I-K) Ouabain-sensitive (OS) and -resistant (OR) Rb⁺ influxes. (L, M) Percentages of cells in L and H fractions (estimated by protein) following 2 h and 4 h incubations in hypertonic media. Data at time zero represent cells in normal RPMI medium. The means $\pm$ SEM were calculated from ≥ 3 independent experiments (except for the 4 h point in 150 mM sucrose, which was tested only once). The data in panels D, H and M were obtained in different series of experiments than the data in the rest of the other panels.

K⁺ content, as well as an increase in Na⁺ content, has been previously observed in U937 cells where apoptosis was induced, in particular, by staurosporine [26]; thus, the ion composition of the H subpopulation is clearly indicative of apoptosis.

The data presented in Table 1 allow semi-quantitative estimates of the contribution of monovalent ions to the total increase of cellular osmolytes responsible for RVI. Table 1 shows that within 2 h of hypertonic challenge one third to one half of the increase in total intracellular osmolarity was due to K⁺ and about $\frac{3}{4}$ due to the sum of K⁺ and Cl⁻. The impact of the changes in Na⁺ content at this time point was small and not identical in different experiments.

DMA+DIDS effect

The RVI response was abolished by DMA+DIDS (DD) used to block the hypertonicity-induced cation channels (HICC) and Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers that are considered key players in RVI in many cell types [27, 33-37]. Indeed, U937 cells incubated in hypertonic medium with 150 mM sucrose in the presence of DD for 2 h formed a single band, as did

Media	RPMI + 200 mM sucrose			RPMI + 300 mM sucrose		
Incubation time	0.5 h	2 h	Difference 0.5-2 h	0.5 h	2 h	Difference 0.5-2 h
K ⁺	731 ± 17 (5)	790 ± 16 (21)	59*	756 ± 29 (8)	880 ± 30 (8)	124*
Na ⁺	239 ± 12 (3)	259 ± 16 (17)	20	167 ± 12 (9)	168 ± 13 (9)	1
Cl ⁻	272 ± 13(12)	368 ± 18 (12)	96*	n.d.	n.d.	n.d.
Water	2.93 ± 0.07 (4)	3.30 ± 0.03 (7)	0.37	2.31 ± 0.02 (3)	2.71 ± 0.02 (3)	0.40
Total osmolytes	1494	1683	189	1409	1653	244

Table 1. Changes in ion and water content associated with RVI in U937 cells incubated in hypertonic media with 200 and 300 mM sucrose. Means ± SEM (number of determinations in parentheses) of values combined from the measurements on fractionated and whole populations (which is acceptable owing to the negligible proportion of H cells during the first two hours of incubation). Ion and osmolyte content is provided in $\mu\text{mol per g}$ of cell protein, and cell water expressed in ml per g of cell protein. Total content of cell osmolytes was calculated by multiplying the water content by the osmolarity of the external medium. The value of Cl^- content at time point 0.5 h was taken equal to its value at time zero. * ($P < 0.03$) indicates statistical significance

Fig. 2. Changes in intracellular Cl^- content caused by hypertonicity. The data at time zero correspond to cells in isotonic RPMI medium. The measurements were done on cells that were not fractionated in Percoll. The values represent the means ± SEM based on 3–5 independent experiments.

Fig. 3. Effect of DMA+DIDS (DD) on cell buoyant density, cell water and K^+/Na^+ content. Cells were incubated in RPMI+150 mM sucrose for 2 h; DMA (0.05 mM) and DIDS (0.5 mM) were added simultaneously with sucrose. The means ± SEM are shown for 3 (–DD) or 2 (+DD) experiments.

untreated cells following 0.5 h incubation in hypertonic medium without blockers. The cell density in this band was similar to the density of the H fraction in the absence of DD. Thus DD abolished both the changes in cell density and in intracellular K^+ content associated with RVI (Fig. 3B). The currently accepted model holds that RVI starts with Na^+ entry and the accumulated Na^+ is exchanged thereafter for K^+ via the Na^+/K^+ pump [35, 38]. This scheme seems to be applicable to U937 cells. It should be noted that no consistent increase in Na^+ content was observed during the time interval 0.5–2 h, whereas the increase in K^+ content was significant (Fig. 1E–H, Table 1). Interestingly, DD abolished the decrease in Na^+ content in cells of the L subpopulation relative to that in H cells, as shown in Fig. 3. No more than a slight effect of DD on the cell buoyant density and the monovalent cation content was found in U937 cells incubated in standard normotonic RPMI medium (data not shown). The NKCC inhibitor, 50 μM bumetanide, was without effect on the RVI in our experiments.

The long-term cell shrinkage seen as a slowly increasing cell density reflected AVD. As shown earlier apoptosis of U937 cells induced by staurosporine is characterized not only by a decrease in cell density and K^+ content but also by inhibition of the sodium pump, i.e. by a decrease in ouabain-sensitive Rb^+ influx [26, 29, 30, 39]. A similar decrease in ouabain-

Fig. 4. Microscopy and morphometry of U937 cells before and following incubation in hypertonic media with 200 mM sucrose. (A-C), Images of cells in culture plates following 1 and 3.5 h incubation. (D), distribution of cells by the area for 0.5 h incubation.

Fig. 5. Cell morphology in light and heavy fractions of U937 cells collected from Percoll density gradient after 2 and 3 h incubation in hypertonic media with 150 mM sucrose. (A) Percentage of cells of different types. Warped cells were discriminated by having roundness less than 0.8 (round is shape descriptor in results menu of ImageJ 1.43u). Means $\pm$ SEM for 5-10 microscopic fields obtained in 1-2 separate experiments and a total of about 200-300 cells. (B-D) Fluorescent images of cells stained by AO (green, orange in electronic version) and EB (red in electronic version) acquired on a Leica TCS SL confocal microscope.

sensitive Rb^+ influx was observed in H subpopulation of U937 cells incubated for sufficiently long time in hypertonic media (Fig. 1I-K).

Microscopy

Under transmission or fluorescent microscope intact U937 cells appeared as a uniform population of relatively round cells (Fig. 4A). During the first 0.5-1 h of incubation in hypertonic media, the cell morphology remained unchanged (Fig. 4B). Strong initial osmotic

shrinkage of round cells placed in a hypertonic solution was revealed by morphometric analysis (Fig. 4D). However, minor changes in cell size associated with RVI and AVD were difficult to analyze by this method (Fig. 4C).

Long-term morphological alteration of cells in hypertonic media occurred asynchronously, and the cell population turned out to be heterogeneous. At least four morphologically distinct types present in all populations could be identified. Examples of these variants are marked in Fig. 5 as normal (*n*), warped without chromatin condensation (*w*), apoptotic with condensed chromatin (*a*), and EB-positive, or dead (*d*).

Fig. 5A shows changes in the percentage of cells of different types following 2 and 3 h incubation in hypertonic RPMI media with 150 mM sucrose. A similar time course of morphological changes, but on a different time scale, was observed in hypertonic media with 200 and 300 mM sucrose, as well as with 100 mM NaCl (images are not shown).

Most importantly, the subpopulation of heavy cells separated in Percoll density gradient, H-subpopulation, contained predominantly cells with condensed chromatin, which is a highly specific marker of apoptosis. This can be seen in Fig. 5A by comparing proportions of different cells following 3 h incubation in H and L subpopulations. Cell shrinkage and chromatin condensation occurred in parallel, supporting the assumption that long-term shrinkage of U937 cells treated with hypertonic solution reflects AVD. Cells with condensed chromatin often, but not always, lost their round shapes. Warped cells did not exhibit chromatin condensation (Fig. 5B-D). Interestingly, the number of the warped cells increased in the L subpopulation and decreased in the H subpopulation inversely to changes in the fraction of apoptotic cells with condensed chromatin (Fig. 5A). Most of the cells in L subpopulation were round and resembled untreated cells (Fig. 5C). Changes in relative numbers of different cell types in samples treated with DD (not shown) confirmed that in this case the percentage of apoptotic cells is increased due to the absence of the L population.

Discussion

As shown in a wide variety of cells employing a variety of experimental approaches, hypertonic stress causes apoptosis [1, 2, 4-18]. It is widely agreed that AVD is one of the hallmarks of apoptosis that precedes other apoptotic markers, e.g. caspase activation, cytochrome c release from mitochondria, DNA fragmentation and chromatin condensation [19-25, 27, 40]. Long-term volume decrease after the initial apoptotic shrinkage was found in Ehrlich ascites cells placed in a hypertonic medium [41, 42]. This effect may be interpreted as the AVD response. A decrease in HL-60 cell volume relative to the initial value following 8 h incubation in a hypertonic medium with 300 mM mannitol has also been reported [13]. Changes in the cell volume of apoptotic human glioblastoma D54-MG caused by various inducers, including hypertonic medium with 300 mM mannitol were reported by Ernest et al. [16].

The RVI response to hypertonic cell shrinkage is a well studied phenomenon [12, 27, 37, 43-46]. The magnitude of the initial cell shrinkage and the subsequent volume increase found in our experiments with U937 cells by using cell density measurement are of the same order of magnitude as reported for other cells using the Coulter electronic sizer and other methods. Our data show that U937 cells respond to hypertonic shrinkage without preliminary incubation in hypotonic media. RVI in U937 cells was associated with the increase in intracellular K^+ rather than Na^+ content. This contrasts the Na^+ increase reported for RVI in HeLa cells [47]. We have also observed that inhibition of Na^+/H^+ and Cl^-/HCO_3^- exchangers or HICC channels abolishes RVI in U937 cells, as it does in many other cell types [27]. The long-term decrease of the cell volume following the initial RVI in hypertonic media may have gone unnoticed in previous studies probably because the experiments were not carried out long enough. By extending observation time to 2-4 h we were able to detect the transition from RVI to AVD in U937 cells. Thus, U937 cells appear to be a useful model to study the switch of cellular volume regulation from RVI to AVD.

The relationship between RVI and AVD was studied in detail in HeLa cells treated by various combinations of hypertonicity and traditional apoptosis inducers, such as staurosporine, TNF- α , FasL etc [47-50]. It was shown, in particular, that suppression of RVI in HeLa cells by HICC, NHE and $\text{Cl}^-/\text{HCO}_3^-$ blockers leads to AVD following hypertonic shrinkage [48]. The conclusion made from these studies was that dysfunction of RVI is a prerequisite for the induction of apoptosis.

Our experiments showed that apoptosis may develop in cells that were initially capable of the RVI response; eventually, RVI gave way to AVD. Therefore, it is not the lack of RVI that triggers apoptosis (accompanied by AVD at a certain stage). Obviously, RVI and AVD cannot be taking place at the same time; it is likely that hypertonic stress initiates both RVI and AVD, but the latter develops slower and becomes detectable at a relatively late stage. The molecular mechanisms underlying the “tug-of-war” between AVD and RVI in HeLa cells have been reported recently by Subramanyam et al. [47], and similar considerations may apply to U937 cells.

With regard to the interplay between RVI and apoptosis, two possibilities should be considered. The first one is that hypertonicity-induced apoptosis becomes possible only when RVI fails for some reason, thus enabling cell shrinkage. This scenario implies that AVD (either by itself or via accompanying ion changes) serves as the signal that launches apoptosis. The second possibility is that both apoptosis and RVI are independently activated by hypertonic stress, but AVD becomes apparent only at a late stage, when shrinkage overrides the effect of RVI. Admittedly, the results of our study do not allow to unequivocally deciding between these two possibilities, but we lean toward the second one. While in some systems AVD has been shown to be a strict requirement for apoptosis [23, 47], in other cases apoptosis can proceed without any shrinkage [29, 51]. Even a transient swelling during early stages of apoptosis has been reported recently [52]. On the other hand, persistent swelling does not seem to be compatible with apoptosis, but is rather a signature of necrotic cell death, leading to plasma membrane rupture with subsequent tissue inflammation. Accordingly, AVD may be viewed as an evolutionary mechanism to prevent swelling and leakage of intracellular components from damaged cells.

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