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COMMENTARY

A Necessary Role for Cell Shrinkage in Apoptosis

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ABSTRACT. The loss of cell volume is a fundamental and universal characteristic of programmed cell death. However, what was once thought to be a passive, secondary feature of the cell death process has now become an area of research interest. Recent studies have integrated cell volume regulation and the movement of ions with the activation of apoptosis. A dramatic reduction of potassium and sodium concentration has been shown to occur in apoptotic cells that exhibit a shrunken morphology. Furthermore, maintaining the normal physiological intracellular concentration of monovalent ions, particularly potassium, inhibits the activation and activity of the death cascades. Thus, the role ions play during apoptosis is more extensive than just facilitation of the loss of cell volume. In this article, we will review the concepts of cell volume regulation and the loss of volume during apoptosis. Additionally, we will underscore our current understanding of ion movement as it relates to the activation of the cell death process. BIOCHEM PHARMACOL **56**;12:1549–1559, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. apoptosis; cell volume; cell shrinkage; regulatory volume increase; regulatory volume decrease; potassium

Apoptosis, also known as programmed cell death, is a physiological mode of cell death where a population of cells is removed from the body in response to a variety of signals [1]. This form of cell death has become the focus of intense research over the past 20 years, due to its involvement in many aspects of biology ranging from embryogenesis and metamorphosis, to cancer and other human diseases. For example, interdigital cell death leading to the regression of the soft tissue or webbing between embryonic digits has been shown to occur by apoptosis [2]. The regression of the tadpole tail during metamorphosis has also been shown to be mediated by apoptosis [3]. Proper functioning of the immune system requires the removal of autoreactive and non-selected T-cells, which occurs by programmed cell death [4]. Recent studies have also documented the occurrence of apoptosis in human disease. Diseases with excessive apoptosis include AIDS and certain neurodegenerative diseases, while some in which apoptosis is inhibited include cancer and autoimmune disorders (reviewed in Refs. 5 and 6). Thus, an understanding of apoptosis will likely be beneficial to many diverse areas of human health.

Apoptosis is characterized by a distinct set of morphological and biochemical features that differ substantially from those observed during necrosis [1, 7, 8]. Cells swell during necrosis due primarily to an early loss of energy metabolism, which is necessary to maintain ionic homeostasis. This increase in cell volume is observed in both the cytoplasm and organelles; however, there is only a negligible effect on the nucleus. There is random degradation of DNA, RNA, and protein, and the DNA appears as a smear when examined by agarose gel electrophoresis. Cells eventually rupture, causing an inflammatory response. In contrast, apoptosis is universally characterized by cellular shrinkage rather than swelling, with condensation of both the cytoplasm and the nucleus. As with necrosis, there is degradation of DNA, RNA, and protein; however, the DNA is cleaved by endogenous endonucleases at the linker region between adjacent nucleosomes. This yields DNA fragments that are multiples of 180–200 bp DNA fragments, which appear as a ladder-like pattern when visualized by agarose gel electrophoresis. Energy levels remain high during apoptosis until very late in the process. Apoptotic cells do not lyse, but portions of the cell bud off in apoptotic bodies that are phagocytized by neighboring cells. Thus, apoptosis is a very efficient process by which the body can remove a population of cells at a given time or in response to a given stimulus without the activation of an inflammatory response.

One of the most ubiquitous and distinctive features of apoptosis is the loss of cell volume, which is common to all examples of apoptosis, independent of cell type. Although this characteristic of cell death has gone largely unexplored for years, recently the loss of cell volume has been the subject of considerable attention. One of the more critical aspects of cell shrinkage during apoptosis may not be the actual loss of cell volume, but the underlying movement of ions, which may facilitate cell shrinkage. The movement of ions, along with the associated changes in cell volume, has been shown to modify cellular metabolism and gene expres-

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sion in liver cells [9] and to control glutamine and glucose metabolism in lymphocytes and macrophages [10]. Therefore, the examination of ion movement as a mechanism of apoptotic regulation has become a challenging area of study, particularly with regard to its integration of signal transduction pathways.

CONTROL OF CELL VOLUME AND VOLUME REGULATION IN LIVING CELLS

For most cells, intracellular ionic concentrations differ significantly from ionic concentrations outside of the cell. For a cell to maintain homeostasis, no osmotic pressure gradient can exist across the cell membrane; therefore, the concentration of osmotically active solute particles inside the cell must equal that on the outside of the cell. The absence of osmotic pressure on the cell membrane results in no net movement of water into or out of the cell, and thus the cell can maintain a constant cell size. Since the cell contains impermeant or fixed anions, such as proteins and DNA, Donnan equilibrium [11] predicts the passive entry of permeant ions, such as Na^+ and K^+, resulting in a positive intracellular osmotic pressure. This positive pressure would be balanced by the movement of water into the cell, which, in turn, would again lead to the passive redistribution of the permeant ions, and the cycle would repeat, causing the cell to eventually swell and lyse. However, cells can compensate for this colloidal osmotic pressure by the movement of ions, primarily through the Na^+/K^+-ATPase [12], in what has been termed the "pump-leak hypothesis" [13, 14]. This ATP-dependent pump exchanges three intracellular sodium ions for two extracellular potassium ions, causing a net efflux of ions. It is responsible for not only the maintenance of the normal cell volume, but also for the high K^+ and low Na^+ intracellular concentrations observed in a majority of mammalian cells. The Na^+/K^+-ATPase, being electrogenic in nature, also provides a small, unbalanced intracellular charge ratio that accounts for a portion of the negative membrane potential of the cell.

Cell volume control in response to changes in the osmolality of the extracellular environment is achieved by the activation of specific pumps and channels, similar to the mechanisms by which cells control their volume under normal or isotonic conditions [15, 16]. Ions can cross the cell membrane in response to a change in the extracellular environment by one of four general mechanisms: electrodiffusion, cotransport, electroneutral exchange, and electrogenic exchange (i.e. Na^+/K^+-ATPase). These ion transport mechanisms are usually quiescent or are active at only a low level under normal isotonic conditions, but can be stimulated under anisotonic conditions. Cells usually maintain their volume within very narrow limits (approximately 3%); however, certain cell types (liver cells, renal medulla epithelial cells, intestinal mucosa) do encounter extreme conditions of tonicity where their extracellular environment may vary greatly. Thus, volume regulatory mechanisms play a fundamental role in the normal, everyday activity of a cell. Additionally, during pathophysiological disturbances, any cell may be exposed to extreme anisotonic conditions, with the volume regulatory response being a protective mechanism.

Although most cells have the ability to respond to changes in their extracellular environment, the actual mechanisms and ionic pathways involved are just beginning to be understood. Cells initially swell when exposed to hypotonic medium due to an influx of water (Fig. 1, top). Most cells can compensate for this increase in cell volume by the activation of an RVD* response to bring cell volume back to a near normal level. This decrease in cell volume is due to the loss of K^+ and Cl^- ions, with the concomitant movement of water out of the cell, although different cell types use different mechanisms during RVD, including activation of the electroneutral K^+/Cl^- cotransporter, the K^+/H^+ exchanger coupled to a Cl^-/HCO_3^- exchanger, or through the activation of individual K^+ and Cl^- channels (Fig. 1, top).

In contrast to cells exposed to hypotonic medium, those exposed to hypertonic medium initially shrink through the efflux of osmotically obligated water (Fig. 1, bottom). Most cells compensate for this volume change by the activation of an RVI response. There is an initial influx of Na^+ and Cl^- into the cell during this response, with the Na^+ being eventually replaced by K^+ through the action of the Na^+/K^+-ATPase. This results in a net increase of K^+ and Cl^- followed by the movement of water into the cell, so that a near normal cell volume can be achieved. As with RVD, the mechanisms involved during RVI are cell-type specific. These mechanisms include activation of the $Na^+/K^+/2Cl^-$ cotransporter and/or the Na^+/H^+ exchanger coupled to a Cl^-/HCO_3^- exchanger (Fig. 1, bottom).

Lymphocytes are one of only a few cell types in which cell volume regulation has been studied extensively, and are also a model system for the study of programmed cell death. Lymphocytes possess two characteristics important for accurate and precise cell volume measurements: an essentially spherical shape and a non-adherent interaction with substrate. Considerable information exists on volume regulation in the various subtypes of lymphoid cells [17, 18]. When T-lymphocytes are exposed to hypotonic conditions, they show an increase in both K^+ and Cl^- efflux from the cell [17]. This ion loss accounts for the RVD response and returns the swollen cells to a near normal cell volume. In contrast, B lymphocytes show only a modest increase in K^+ efflux under hypotonic conditions, and therefore, very little RVD [17]. This difference in RVD activity is due to a failure of hypotonic conditions to stimulate a loss of K^+ in B lymphocytes. Interestingly, these cells still lose Cl^-, supporting the idea that the loss of K^+ and Cl^- during RVD in lymphocytes is through independent pathways.

T-lymphocytes exposed to hypertonic conditions shrink

* *Abbreviations:* IL-1β, interleukin-1β; Neo, neomycin; RVD, regulatory volume decrease; and RVI, regulatory volume increase.

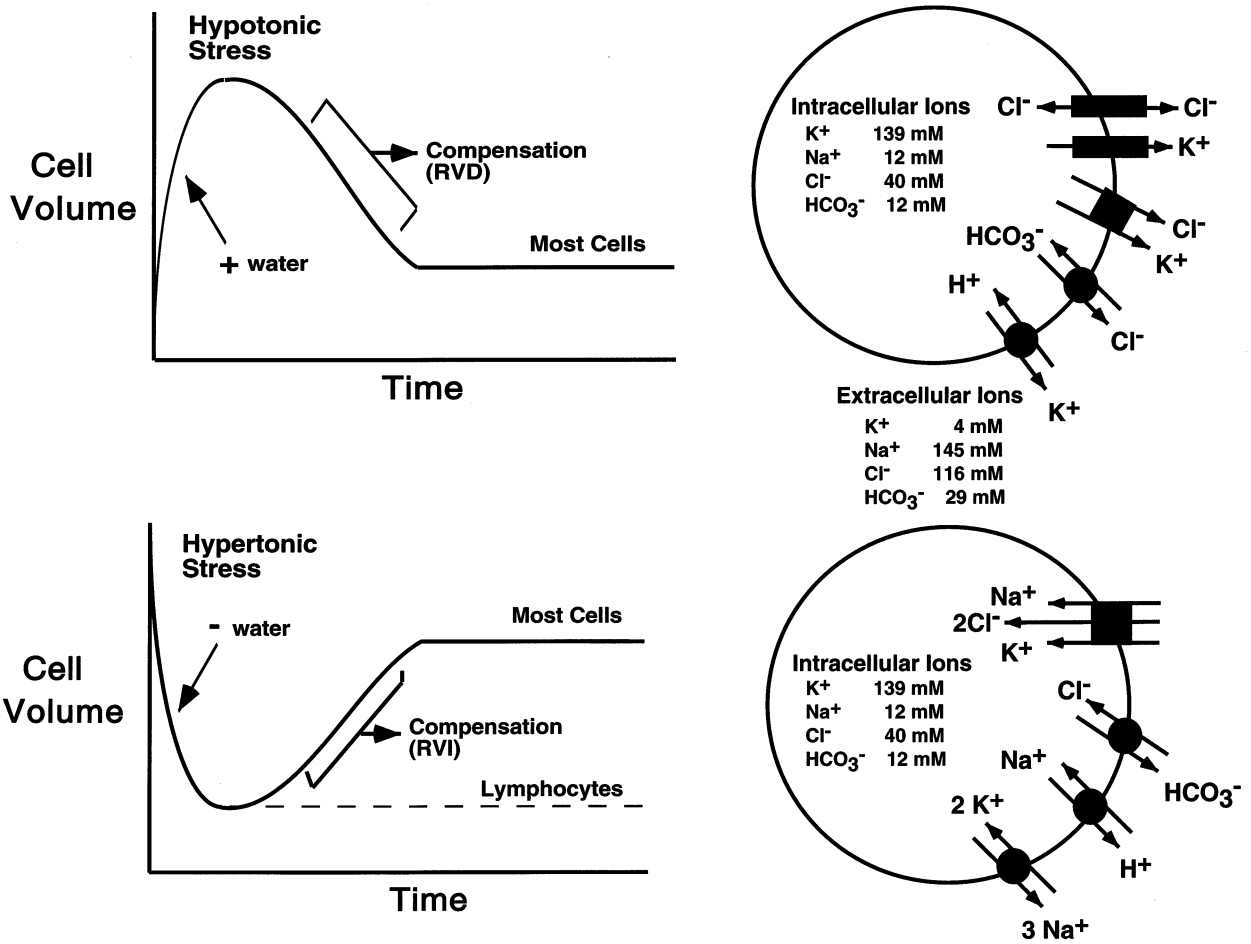

FIG. 1. Response of cells to anisotonic conditions. When cells are exposed to a hypotonic environment, they initially gain water and swell. However, over a period of time, these cells compensate for this increase in cell volume by the activation of an RVD response. This response occurs by the activation of various ion transport pathways including the electroneutral K^+/Cl^- cotransporter, the K^+/H^+ exchanger coupled to a Cl^-/HCO_3^- exchanger, or through the activation of individual K^+ and Cl^- channels, which, in turn, allows for the movement of water from the cell. In contrast, when cells are exposed to a hypertonic environment, they initially lose water and shrink. These cells can compensate for this loss in cell volume by the activation of an RVI response. This response allows for the movement of water into the cell through the activation of the $Na^+/K^+/2Cl^-$ cotransporter and/or the Na^+/H^+ exchanger coupled to a Cl^-/HCO_3^- exchanger. Additionally, the Na^+ is replaced with K^+ through the constitutively active Na^+/K^+-ATPase.

and remain shrunken ([19, 20]; Fig. 2, top). This absence of RVI in T-cells is not absolute, as Grinstein *et al.* [21] showed that peripheral blood lymphocytes can undergo RVI if first primed by hypotonic conditions. When peripheral blood lymphocytes are equilibrated under hypotonic conditions and then placed into an isotonic environment (which is now hypertonic to the original condition), the cells shrink and regain their cell volume by an RVI response. We have also shown this type of RVI response in S49 Neo thymoma cells using flow cytometry. A typical size distribution of S49 Neo cells cultured in normal or isotonic (300 mOsM) medium is shown on the forward-scatter histogram in Fig. 2 (top, control time 0). When the osmolality is increased by 250 mOsM using mannitol (550 mOsM final), the entire population of cells immediately shrink and remain in this shrunken state over a period of 1 hr, without the activation of an RVI response (Fig. 2, top). When S49 Neo cells are cultured in a hypotonic medium (150 mOsM), they swell and volume-regulate back, via RVD response, to a near normal cell size within 1 hr (Fig. 2, bottom). When these hypotonically treated cells are then placed back into an isotonic medium (300 mOsM), they immediately shrink and volume-regulate back, via RVI response, to a near normal cell size within 1 hr. This secondary RVI response has been shown to occur by the activation of a Na^+/H^+ exchanger coupled to a Cl^-/HCO_3^- exchanger [21]. The exact mechanisms that trigger these exchangers and transporters in lymphocytes are unknown. Additionally, the exact role these volume regulatory mechanisms play in cell shrinkage during apoptosis is also currently unknown.

CONTROL OF CELL VOLUME DURING APOPTOSIS

Cell shrinkage is a classic feature of apoptosis and has been observed from the very first reports on the programmed cell death process [8, 22]. T-lymphocytes have been an excel-

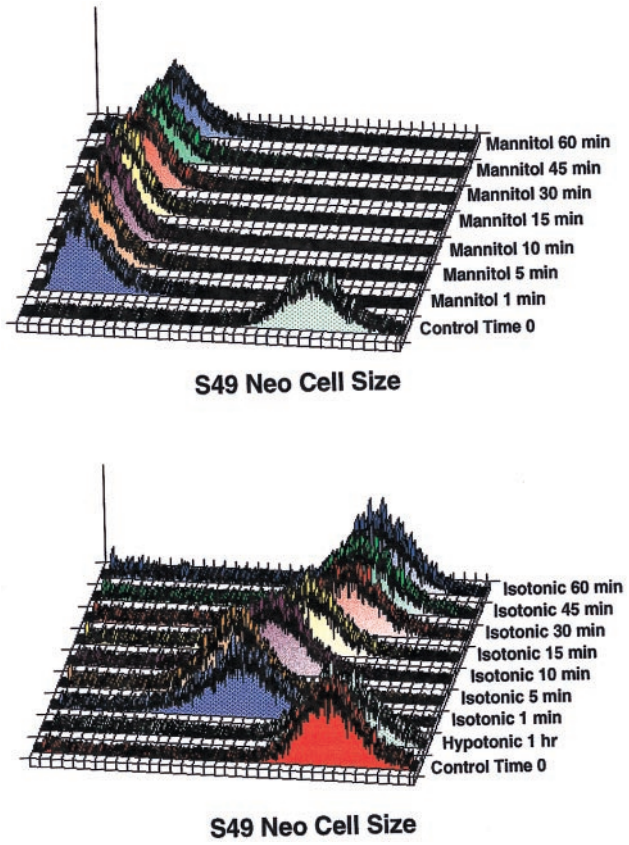

FIG. 2. Demonstration of a secondary RVI response in S49 Neo cells. S49 Neo cells, a murine thymoma cell line, were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 4 mM glutamine, 75 U/mL of streptomycin, and 100 U/mL of penicillin at 37°, 7% CO_2 atmosphere. Cell size was determined by flow cytometry using a Becton Dickinson FACSort and examination of the forward-scatter light property of 10,000 individual cells at each given time point. Forward-scattered light is a direct measure of cell size, with cells of a larger size having an increase in forward-scattered light, and smaller cells having a decrease in forward-scattered light. In the top panel, control cells (cells cultured in RPMI medium) are shown as a single population of cells on a forward-scatter histogram. When the RPMI 1640 medium is supplemented with 250 mM mannitol (thus increasing the osmolality from the normal 300 mOsM to approximately 550 mOsM), the entire population of cells shrink (shown as a shift to the left on the forward-scatter histogram). These cells, maintained in this hypertonic environment, remain shrunken over a period of 60 min, and show no indication of an RVI response. In the bottom panel, control cells are shown as a single population of cells on a forward-scatter histogram. When these cells are placed in a hypotonic environment, where the osmolality of the RPMI 1640 medium was reduced to approximately 150 mOsM by the addition of water, they swell and volume-regulate back to a near normal cell size within 1 hr. When these cells are placed back into the normal RPMI 1640 medium (which is now hypertonic to the regulated cells) after this RVD response, they shrink. However, over a period of 60 min they volume-regulate by a secondary RVI response back to a near normal cell size.

lent model system for studying programmed cell death because of their marked susceptibility to undergo apoptosis in response to a variety of agents, including glucocorticoids. Glucocorticoid-induced cell size changes in thymocytes were investigated as early as 1981 [23]. Treatment of freshly isolated rat thymocytes with dexamethasone resulted in the progressive appearance of shrunken cells in a concentration-dependent manner, and also required protein synthesis. Electronic sizing techniques showed that these shrunken, apoptotic cells had a mean diameter of 4.6 μm compared with a diameter of 5.2 μm for normal cells. Interestingly, no distinct intermediate-sized population of cells was observed.

Isopyknic centrifugation was also used in early studies to ascertain whether differences in the buoyant density exist between normal and apoptotic cells [24, 25]. The buoyant density of untreated rat thymocytes averaged 1.075 g/mL, whereas the population of methylprednisolone-treated thymocytes after 1 hr had two distinctly different densities: one similar to the untreated population of cells, and a second with increased buoyant density [24]. Percoll separation showed that the more dense cells consisted almost entirely of apoptotic cells, whereas the less dense cells were similar to the untreated population. This high density stage was transient and did not persist during extended incubations (where chromosomal condensation, characteristic of apoptosis, was observed). Based on these findings, these investigators concluded that the cell density increase observed in apoptotic cells must be due to a loss of water.

Benson *et al.* [26] recently characterized the loss of cell volume in an established T-cell line (CEM-C7A) during dexamethasone-induced apoptosis. Treatment of CEM-C7A cells for 48 hr with dexamethasone resulted in a 42% decrease in cell volume relative to untreated cells. Buoyant density measurements revealed no change during the first 24 hr of dexamethasone treatment, but a less dense fraction appeared at a later time. The authors' interpretation of these results is that cell volume decreased in two phases. The first phase was suggested to be mediated not only by the loss of intracellular ions and water, but also by the export of cytoplasmic contents, and, therefore, maintenance of a normal cell density. The second phase was proposed to represent a loss of degraded macromolecules from the cell during apoptotic fragmentation. The idea that a loss of cell volume during apoptosis occurs in stages has been suggested previously by other investigators [27–29]. For example, in a study involving radiation-induced apoptosis of rat thymocytes, cell shrinkage was reported to occur initially as a rapid event, with cell size decreasing to approximately 75% of its original volume. Subsequently, a gradual decrease in cell size occurred over a period of several hours, with a resultant reduction in cell volume to approximately 57% of the original volume. These authors concluded that most of the material lost during both stages of cell shrinkage was water, and not cytoplasmic or cellular contents. These studies suggest that several stages of cell volume loss occur during apoptosis and conclude that this shrinkage is due primarily to the loss of water.

Human eosinophils also lose cell volume during apoptosis induced by cytokine withdrawal [30]. The viability of purified human eosinophils was reduced to 24% after 48 hr

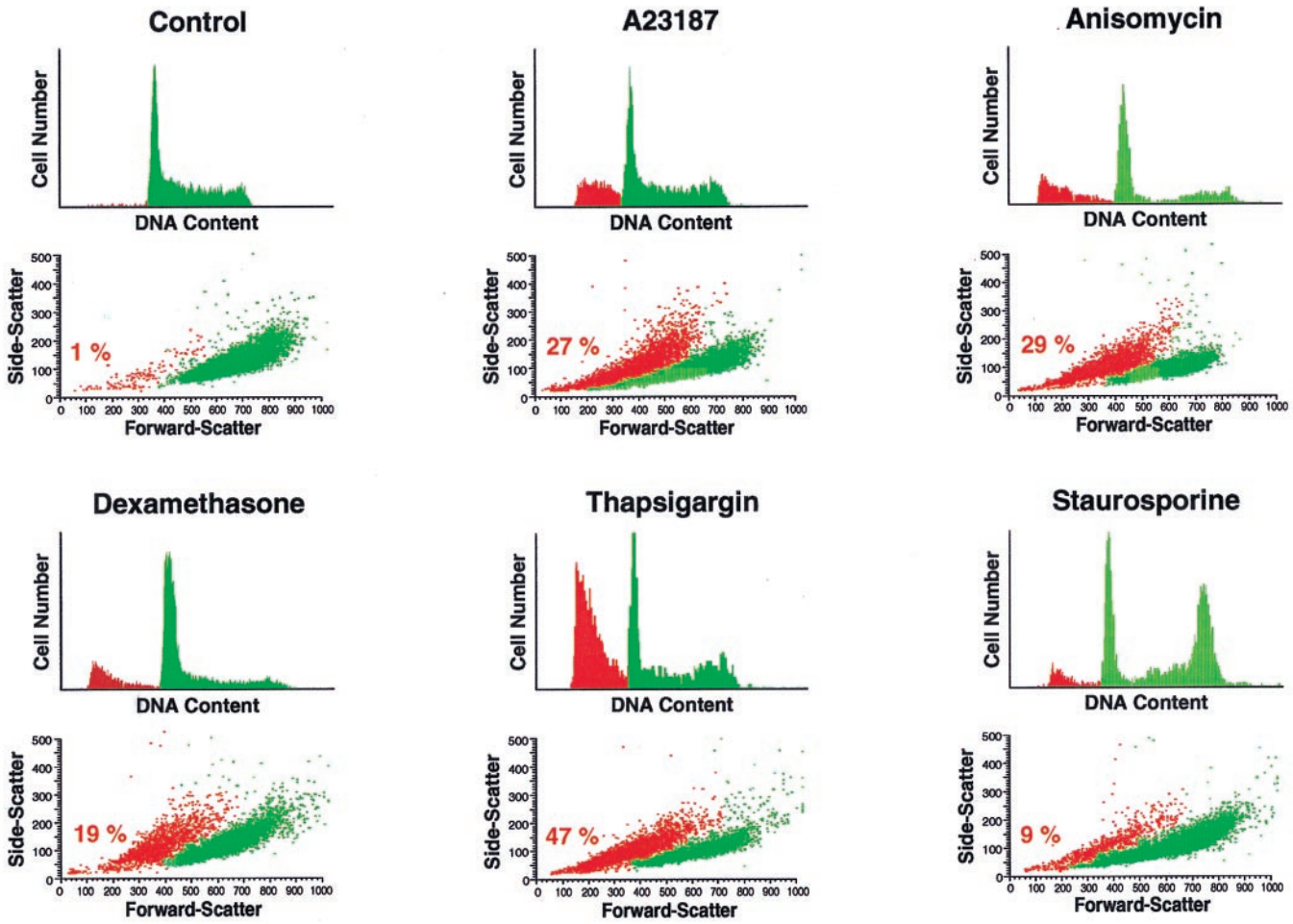

FIG. 3. Relationship between cell volume and DNA degradation. S49 Neo cells were treated with various apoptotic agents, fixed, and then examined by flow cytometry. Analysis of the DNA content and light-scattering properties of 7500 control cells showed a normal DNA content profile and a single population of cells on a forward-scatter versus side-scatter dot plot. Gates set on a propidium iodide based area versus width dot plot were used to separate and analyze the DNA content in this sample to determine the position of the cells with various DNA integrity on the light-scatter dot plot by multi-color analysis. An initial gate was set on the single population of control cells observed on the area versus width dot plot. A second gate was set for cells that have a reduced area and width. These gates were then also used to examine cells treated with the various apoptotic agents. Analysis of the DNA content in the control sample showed that cells with a normal DNA profile had a large cell size (shown in green), while the small proportion of cells in the subdiploid region of the DNA content histogram, representing degraded DNA, had a small or shrunken cell size (shown in red). The number in red shows the percent of shrunken cells with degraded DNA. Upon treatment with various apoptotic stimuli, the number of cells comprising the subdiploid region of the DNA content histogram increased and directly correlated to the position of the smaller size cells on the forward-scatter versus side-scatter dot plots. Therefore, only the shrunken population of cells contained DNA, which had been degraded. Data shown are representative of three identical experiments. Reprinted with permission from *J Biol Chem* 272: 32436–32442, 1997. Copyright (1997) The American Society for Biochemistry and Molecular Biology [Ref. 38].

in the absence of cytokine, with over 70% of these cells showing a decrease in cell size. Electronic sizing of these apoptotic cells showed an average 63.2% reduction in cell volume, which is even greater than the 43% reported for irradiated rat thymocytes as described above, further demonstrating the cell-type based variation in size that can occur during apoptosis. A clear bimodal size distribution was observed, such that the eosinophils appeared either normal or shrunken, with few cells in an intermediate state. This study also examined volume reduction in neutrophils, which spontaneously undergo apoptosis in culture. Interestingly, after 24 hr in culture, cells that comprised the smaller-sized population had only a 30% decrease in cell volume, and overlapped the volume distribution of the normal or non-apoptotic cells. Although there may be cell-type specific pathways accounting for reduced volume during early stages of apoptosis, the loss of cell volume still remains a common feature of programmed cell death in a wide variety of systems.

Flow cytometry has become an important tool for studying apoptosis, particularly with non-adherent cells. Our laboratory was the first to show the effects of glucocorticoid-induced apoptosis on rat thymocyte DNA at the single cell level by flow cytometry [31]. Isolated thymocytes from

adrenalectomized rats treated with dexamethasone *in vitro* and stained with acridine orange showed a reduction in DNA fluorescence in a subpopulation of cells. This indicated a loss of cellular DNA that correlated directly with the kinetics of DNA degradation observed *in vivo* [32]. Swat *et al.* [33] also used flow cytometry to examine apoptosis in immature thymocytes. When freshly isolated mouse thymocytes were compared with thymocytes cultured for a period of 24 hr, elevated numbers of the cultured thymocytes had a decrease in forward-scattered light (reduced cell volume) and an increase in side-scattered light (increased cell density). Treatment of HL-60 cells with the topoisomerase inhibitor camptothecin also resulted in a decrease in forward-scattered light, which occurred as early as 2 hr after treatment [34]. Unlike the increase in side-scattered light observed in 24-hr cultured primary thymocytes, camptothecin-treated HL-60 cells exhibited a decrease in side-scattered light after 3 hr of treatment. Darzynkiewicz and colleagues [34] hypothesized that the increase in cell density observed after a 24-hr culture of primary thymocytes probably reflects chromatin condensation and nuclear fragmentation of the cell. Therefore, it appears that T-cells may initially undergo a decrease in cell density, and then increase their cell density concurrent with the other morphological characteristics of apoptosis. The kinetics of programmed cell death also have been studied by flow cytometry in mature human T-cells [35]. Activation of apoptosis in these cells by either ionomycin, the mitogenic lectin phytohemagglutinin or anti-T-cell receptor monoclonal antibodies showed that although the timing of apoptotic induction varied between these agents, a loss of cell volume was always observed prior to DNA fragmentation.

We recently investigated the role of cell volume loss in inducing apoptosis in immature thymocytes [36]. As mentioned above, thymocytes do not initially respond to a loss in cell volume with an RVI response. Exposure of S49 Neo cells to hypertonic conditions, using either mannitol, sucrose, or NaCl, resulted in a very rapid loss of viability. The viability loss observed in response to increased extracellular osmolality was accompanied by a sustained decrease in cell volume. In addition, internucleosomal DNA cleavage and apoptotic body formation, both recognized characteristics of apoptosis, were also observed. Interestingly, when S49 Neo cells were treated for only 1 hr under hypertonic conditions and returned to an isotonic environment, they failed to undergo apoptosis. However, if the hypertonic treatment was extended for 2 hr, the entire population of cells were programmed to die via apoptosis (Bortner CD and Cidlowski JA, unpublished results). These data suggest that signals in addition to cell shrinkage are needed to activate apoptosis. Cell death induced by hypertonic conditions also does not require protein synthesis, in stark contrast to dexamethasone-induced apoptosis, and suggests that these cells contain all of the necessary proteins required to execute the apoptotic process. Treatment of S49 Neo cells with hypotonic conditions for a similar period of time resulted in cell swelling but did not induce apoptosis, also suggesting that a specific reduction in cell volume was required to trigger programmed cell death. We extended these studies to include additional thymic lymphoid cells, which do not demonstrate an initial RVI response, including CEM-C7 cells and primary thymocytes, and observed a similar induction of apoptosis in response to hypertonic conditions. When non-lymphoid cells (COS, HeLa, GH_3, and L-cells) were exposed to the same hypertonic conditions, an initial decrease in cell volume was observed followed by an RVI response with no subsequent apoptosis. Therefore, the presence of a volume regulatory response may act as a first line of defense to protect cells against programmed cell death. These results demonstrate that cell volume plays a crucial role during apoptosis, and suggest that mechanisms that control cell volume must be either inhibited or overridden for programmed cell death to ensue.

Flow cytometry of cells after staining with various fluorescent dyes allows for the simultaneous assessment of light-scattering properties along with other parameters, such as DNA content, caspase activity, and mitochondrial membrane potential [37]. A decrease in DNA content, as ascertained by propidium iodide staining, has been correlated with apoptotic fragmentation of the DNA [31], and can be directly related to the size of individual cell populations by flow cytometry. Examination of dexamethasone-treated S49 Neo cells by flow cytometry revealed that only cells exhibiting a loss in cell volume had DNA that was degraded [36]. This relationship between the loss of cell volume and DNA degradation has also been observed with other apoptotic inducing agents and in a variety of lymphoid cells [38]; Fig. 3). When S49 Neo cells were treated with known apoptotic agents such as dexamethasone, A23187, thapsigargin, anisomycin, or staurosporine, a subpopulation of cells was observed, which had degraded their DNA. When these cells were examined on a forward-scatter versus a side-scatter plot to assess cell size, only cells that had degraded their DNA comprised the shrunken population of cells. This suggests an important relationship between the loss of cell volume and activation of the nucleases associated with apoptosis. Therefore, knowledge of how cell volume loss occurs and how cell shrinkage relates to other characteristics of apoptosis would likely further our understanding of the entire programmed cell death process.

POTASSIUM-MEDIATED LOSS OF CELL VOLUME DURING APOPTOSIS

Over the past several years, ion channels, particularly potassium channels, have become an area of active research due to their presence in all mammalian cells and their involvement in many normal cellular processes. Potassium channels represent a very diverse group of ion channels and can be categorized according to their number of transmembrane segments, gating properties, or sensitivity to various potassium channel antagonists. Data accumulating from the sequencing of the *Caenorhabditis elegans* genome has

shown that the diverse array of potassium channels is not limited to mammalian cells, but also extends to other eukaryotes. Over forty different potassium channel genes, representing eight conserved multigene families, have been identified thus far in only the first third of the genome [39]. This large number of genes suggests that potassium channels play an extensive role in cellular biology. Potassium channels, along with potassium transporters and exchangers, have a much more diverse function than was initially proposed when a potassium channel was first cloned from *Drosophila* over a decade ago [40, 41]. The role of potassium channels has been extended from their function in excitable cells, to shaping of passive ionic properties of non-excitable cells, and involvement in many disease states including hypertension, asthma, urogenital malfunction, and various cardiac anomalies [42].

The role of potassium channels in cell activation, mitogenesis, cytotoxicity, and volume regulation has been studied extensively in lymphocytes (reviewed in Refs. 43 and 44). Several types of voltage-gated K^+ channels, along with Ca^{2+}-activated K^+ channels, have been observed in lymphocytes; however, their distribution varies among the numerous subsets of T-cells. Recently, it has been suggested that potassium loss may underlie the cell shrinkage associated with apoptosis [26, 30, 36, 38, 45]. Our laboratory has shown that both the K^+ and Na^+ concentrations in lymphocytes treated with several apoptotic agents decrease dramatically in the viable, shrunken population of cells [38]. We have also demonstrated that apoptosis is enhanced under conditions where the intracellular K^+ concentration is diminished (Fig. 4). Jurkat cells, a human leukemic cell line, cultured under hypotonic conditions to reduce the level of intracellular K^+ (via RVD response) undergo apoptosis approximately 85% faster upon anti-Fas stimulation than anti-Fas-treated Jurkat cells cultured in normal medium (Fig. 4, A and B). This result suggests that the loss of K^+ alone is not the trigger for apoptosis, since cells cultured under hypotonic conditions, where the intracellular potassium concentration is reduced, do not undergo cell death in the absence of an apoptotic stimulus. However, this initial loss of intracellular K^+ does potentiate cell death initiated by treatment with an apoptotic agent. In contrast, when Jurkat cells were cultured in medium where the normal concentrations of Na^+ and K^+ were switched to eliminate the normal K^+ concentration gradient, apoptosis was inhibited, suggesting that the loss of potassium is a key event in the cell death process (Fig. 4C). In support of these findings, McCarthy and Cotter [46] also observed a decrease in both K^+ and Na^+ concentration in HL-60 cells induced to undergo apoptosis via UV-irradiation and cytotoxic stimulation. Similarly, apoptosis in mouse neocortical neurons was associated with an early enhancement of delayed rectifier current and the loss of intracellular K^+ [47]. In several studies, the use of potassium channel blockers, such as 4-aminopyridine [30], tetraethylammonium [47], and tetrapentylammonium [46], was shown to inhibit cell shrinkage; however, it is unclear if this inhibition blocked cell death or just delayed the process. In a murine model of septic liver failure, the release of tumor necrosis factor, early apoptosis, and subsequent necrosis was shown to be inhibited by quinine, a potent K^+ channel blocker [48]. None of these potassium channel blockers inhibited the loss of cell volume in our studies (Bortner CD and Cidlowski JA, unpublished results), suggesting the presence of cell-type specific pathways of K^+ efflux that may be related to the specific RVD responses. Interestingly, several K^+ ionophores, such as (−)-cromakalim, valinomycin, and beauvericin, have been shown to enhance or cause apoptosis in general systems including lymphocytes [47, 49], supporting our observation that reduced intracellular potassium concentrations can enhance programmed cell death [38].

DOES POTASSIUM DO MORE THAN REGULATE CELL VOLUME?

While the role potassium plays in the loss of cell volume during apoptosis seems obvious, the role it may play in other aspects of the programmed cell death process is less apparent. Recently, several laboratories including our own have begun to examine how intracellular levels of potassium may control and repress apoptosis [50, 51]. Addition of low concentrations of staphylococcal α-toxin to T cells was shown to create small pores in the cell membrane and induce internucleosomal DNA cleavage, characteristic of apoptosis [50]. These small pores allow the passage of monovalent ions only, such as potassium and sodium, whereas the passage of larger ions and molecules, such as calcium and propidium iodide, is inhibited. Apoptosis could be induced by allowing potassium to run down its concentration gradient, thus decreasing the intracellular potassium concentration. In contrast, addition of higher concentrations of α-toxin creates larger pores in the cell membrane, resulting in a rapid loss of ATP and no DNA degradation, suggesting the importance of ATP in controlling the movement of ions during apoptosis. Interestingly, when T-cells were cultured in sodium-free buffer with low doses of α-toxin, the cells neither degraded their DNA nor lost viability. These studies suggest that both the loss of potassium and the presence of sodium were necessary for DNA degradation. However, the exact role these ions may play in nuclease activation and DNA fragmentation during apoptosis was not clear at that time.

We have also shown recently that normal intracellular concentrations of potassium directly inhibit nuclease activity during apoptosis *in vitro* and *in vivo* [51]. This inhibitory effect on nuclease activity could be mimicked by other monovalent ions such as sodium and cesium, suggesting that the regulatory nature of ions during apoptosis does not rely on a single ion, but probably depends on the overall ionic strength in the cell. The importance of potassium in nuclease inhibition likely lies in the fact that it is the only ion present at high concentrations in the cell. For example, caspase-3-like activation during apoptosis is dependent

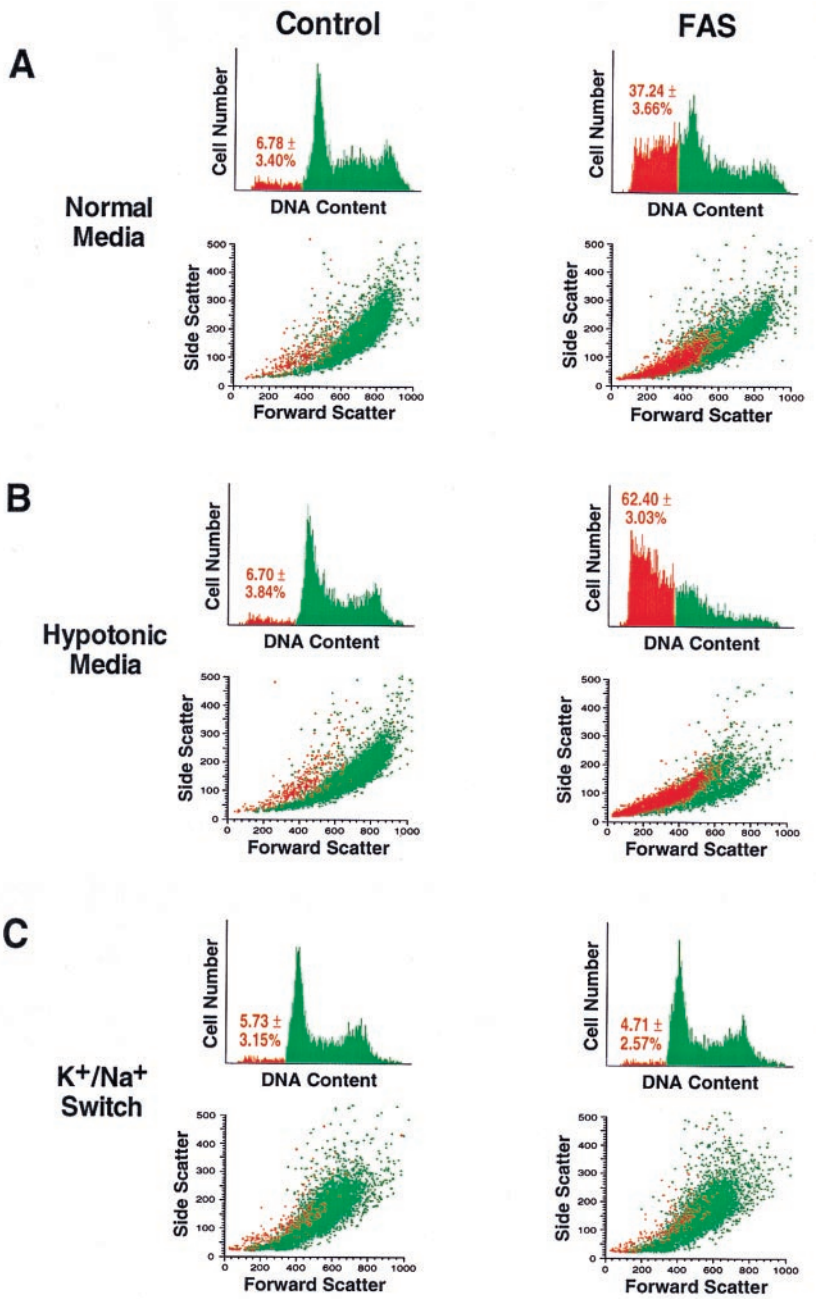

FIG. 4. Jurkat cells treated without and with anti-Fas antibody under normal, hypotonic, or increased extracellular potassium conditions. Plots shown are representative of a single study, and the percentages are the averages of 4 independent experiments. (A) Jurkat cells treated with anti-Fas antibody for 5 hr showed an increase in the percentage of cells (37%) that degraded their DNA compared with the control cells (7%) along with the concomitant increase in the number of shrunken cells. (B) Under hypotonic conditions, where the cells initially swell and then regulate their volume by the loss of potassium and chloride, the percentage of anti-Fas-trated Jurkat cells that have degraded DNA in 5 hr was increased by 85% [62% DNA degradation in the anti-Fas-induced hypotonically treated cells compared to 37% DNA degradation in the anti-Fas-induced cells under normal (isotonic) conditions]. This increase in DNA fragmentation was attributed to the hypotonic cells having a lower potassium content during the anti-Fas treatment. (C) When the normal concentrations of sodium and potassium were switched in the external environment, limiting the amount of potassium that can be loss during apoptosis, the loss of cell volume and DNA degradation induced by anti-Fas treatment were not observed. Reprinted with permission from *J Biol Chem* 272: 32436–32442, 1997. Copyright (1997) The American Society for Biochemistry and Molecular Biology [Ref. 38].

upon the loss of intracellular potassium, although the activity of the mature caspase-3-like enzymes is not affected by the level of potassium in the cell [51]. This observation is supported by an earlier study demonstrating processing of pro-IL-1β to mature IL-1β in human monocytes by the interleukin converting enzyme (ICE; also known as caspase 1) via intracellular potassium depletion [52]. Additionally, hypotonic conditions (where K^+ and Cl^- ions are lost from

the cell) were shown to promote the release of mature IL-1β from lipopolysaccharide-activated monocytes, while hypertonic conditions blocked this ATP-induced posttranslational processing reaction [53].

Further evidence of a role for ionic regulation of apoptosis comes from studies done on the pro- and anti-apoptotic Bcl-2 family of proteins. Gilbert *et al.* [54] demonstrated that overexpression of Bcl-2 in a human B-cell lymphoma cell line and in HL-60 cells affects the cellular membrane potential. Cells expressing Bcl-2 were hyperpolarized compared with control cells, and this more negative membrane potential correlated with increased radioresistance. X-ray crystallographic and NMR examination of the structure of Bcl-X_L, an anti-apoptotic member of the Bcl-2 family, revealed a structure similar to the structure of the pore-forming domains of bacterial toxins [55]. Electrophysiological studies demonstrated that Bcl-X_L forms a pH-sensitive, cation-selective ion channel in synthetic lipid membranes [56]. Additional studies on other members of the Bcl-2 family of proteins, such as Bax and Bcl-2, also showed ion flux or channel-like characteristics of these family members [57]. Interestingly, Bax was shown to display Cl^- selectivity, whereas Bcl-2 was shown to display K^+ selectivity. Therefore, the counter-ion selectivity of these pro- and anti-apoptotic proteins strongly suggests a level of control during programmed cell death related to the movement of ions.

Potassium channels have been proposed to be involved in the proliferation of many cells, including their progression through the cell cycle [58]. Inhibition of K^+ channels with 4-aminopyridine inhibited the proliferation of human myeloblastic ML-1 cells [59] and induced apoptosis in malignant astrocytoma cells [60]. This apparent dependence on potassium for maintenance of the malignant astrocytoma cells is similar to conditions observed for cerebellar granule neurons, which have been shown to undergo apoptosis when cultured in normal potassium concentrations (5 mM) [61, 62]. In contrast, cerebellar granule neurons maintained in high potassium (25 mM) survive, probably through either an influx of Ca^{2+} (reviewed in Ref. 63), or through the activation of various kinases [64].

Seemingly paradoxical data showing an inhibition of voltage-gated potassium channels during apoptosis have been reported recently. Ligation of the Fas receptor in Jurkat cells resulted in inhibition of voltage-dependent n-type K^+ channels (Kv1.3) [65]. Ceramide (a lipid metabolite synthesized upon Fas receptor ligation) and the generation of reactive oxygen species, which are known to be involved in Fas-induced apoptosis, have also been shown to inhibit n-type K^+ channels [66–68]. These studies involving K^+ channel inhibition upon Fas receptor ligation focus only on early cellular changes, and are probably not associated with the K^+-dependent loss of cell volume observed during cell death. Furthermore, it is not clear whether inhibition of these voltage-gated potassium channels is directly involved in apoptosis or is secondary to the generalized process of cell death. Taken together, studies done to date suggest a pivotal role for potassium not only in the maintenance of normal cell homeostasis, but also in the physiologic cell death process known as apoptosis.

SUMMARY

The loss of cell volume is a fundamental feature of apoptosis and is observed in all well-characterized cases of programmed cell death. For many years it has been known that cells can compensate for changes in their cell volume caused by changes in their intracellular and extracellular environment by the activation of various volume regulatory response mechanisms. We have shown that these mechanisms may play a vital role in cell survival during apoptosis as demonstrated by the fact that lymphoid cells, which do not display an RVI response to hypertonic conditions, undergo apoptosis [36]. In contrast, cells that can regulate their cell volume are resistant to hypertonically induced cell death. An interesting question remains as to how these volume regulatory mechanisms are inhibited or overridden, thus allowing apoptosis to occur.

The movement of ions may be a fundamental mechanism of control during apoptosis, such that changes in cell volume in response to ion movement can be a physiological signal to activate events leading to cell death. Apoptosis is a very dynamic process, and the recent discovery of various caspase activities during cell death has also led to a new understanding of the mechanisms underlying the biochemistry of programmed cell death. While the activation of a cascade of caspases is known to occur during apoptosis, the targets or substrates for these enzymes are not completely known. Our work has shown that physiological intracellular concentrations of potassium inhibit the apoptotic machinery, including caspase-3-like activation, and a significant decrease in the overall intracellular ionic strength is required to activate various caspases and nucleases known to be involved in the cell death process [51]. It has not been determined whether caspase activity has a direct effect on various ion channels or transporters. Furthermore, the loss of cell volume may alter gene transcription, the phosphorylation of various proteins, and/or allow for the physical disruption of normal protein–protein interactions to either activate key cell death enzymes or obstruct endogenous inhibitory proteins.

An early movement of ions may also occur, which, in turn, may facilitate a later movement and the loss of cell volume, such that one ion may affect another in a cascade-like response, similar to what is observed for the caspases. One needs to consider the overall effect that such ion fluxes may have on the cell, since an orchestrated movement of ions may occur during apoptosis, and any intervention into this defined movement may disrupt the entire cell death process. In support of this, it has been shown that interfering with the control of ion gradients, and subsequent cellular changes, causes necrosis, not apoptosis, in L cells treated with etoposide (VP-16) or thymidine [45]. Future

studies on ion movement will enhance our understanding of apoptosis.

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