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REVIEW

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Cell volume regulation in immune cell apoptosis

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Abstract The loss of cell volume is an early and fundamental feature of programmed cell death or apoptosis; however, the mechanisms responsible for cell shrinkage during apoptosis are poorly understood. The loss of cell volume is not a passive component of the apoptotic process, and a number of experimental findings from different laboratories highlight the importance of this process as an early and necessary regulatory event in the signaling of the death cascade. Additionally, the loss of intracellular ions, particularly potassium, has been shown to play a primary role in cell shrinkage, caspase activation, and nuclease activity during apoptosis. Thus, an understanding of the role that ion channels and plasma membrane transporters play in cellular signaling during apoptosis may have important physiological implications for immune cells, especially lymphocyte function. Furthermore, this knowledge may also have an impact on the design of therapeutic strategies for a variety of diseases of the immune system in which apoptosis plays a central role, such as oncogenic processes or immune system disorders. The present review summarizes our appreciation of the mechanisms underlying the early loss of cell volume during apoptosis and their association with downstream events in lymphocyte apoptosis.

Key words Apoptosis · Cell volume · Lymphocyte · Ion channels · Glucocorticoids · Fas

Introduction

Prokaryotes and plant and animal cells have developed a number of different cell volume regulatory mechanisms to respond to anisotonic conditions that challenge their normal cellular homeostasis and function. However, the link between cell volume regulation and programmed cell death would be seemingly insignificant except that one of the incipient features of apoptosis is the loss of cell volume (Kerr et al. 1972; Searle et al. 1982; Wyllie 1980). The loss of cell volume observed during apoptosis is in marked contrast to the cellular swelling which occurs during an accidental cell death process known as necrosis (Kerr et al. 1972). During necrosis, the plasma membrane integrity is disrupted by an early loss of ATP that arrests many of the ion transport mechanisms required to maintain a normal cell volume. A necrotic cell loses its capacity to maintain normal plasma membrane osmotic pressure, which results in cell swelling, rupture of the plasma membrane, and leakage of intracellular content. This in turn induces an inflammatory response and damage in the surrounding tissue. In contrast, apoptosis is accompanied by cell shrinkage, and the plasma membrane integrity and cellular energy levels are generally maintained until late in the cell death process, implying that the loss of cell volume is an active process. Eventually, portions of the cell "bud off" in what has been termed apoptotic bodies that preserve surrounding cells from an abrupt immune response. To appreciate the impact that cell shrinkage has during apoptosis, one must first understand the mechanisms by which a cell responds to changes in cell volume.

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Mechanisms of cell volume regulation in mammalian cells: regulatory volume increase and regulatory volume decrease responses

A number of regulatory mechanisms including ion channels, plasma membrane transporters and cytoskeletal reorganization are responsible for maintaining cell volume. In mammals, the kidneys play an indispensable role in maintaining a constant extracellular osmotic environment, such that cells are relatively unaffected by osmotic stress. However, under certain pathophysiological conditions, cells may be exposed to extreme changes in extra-

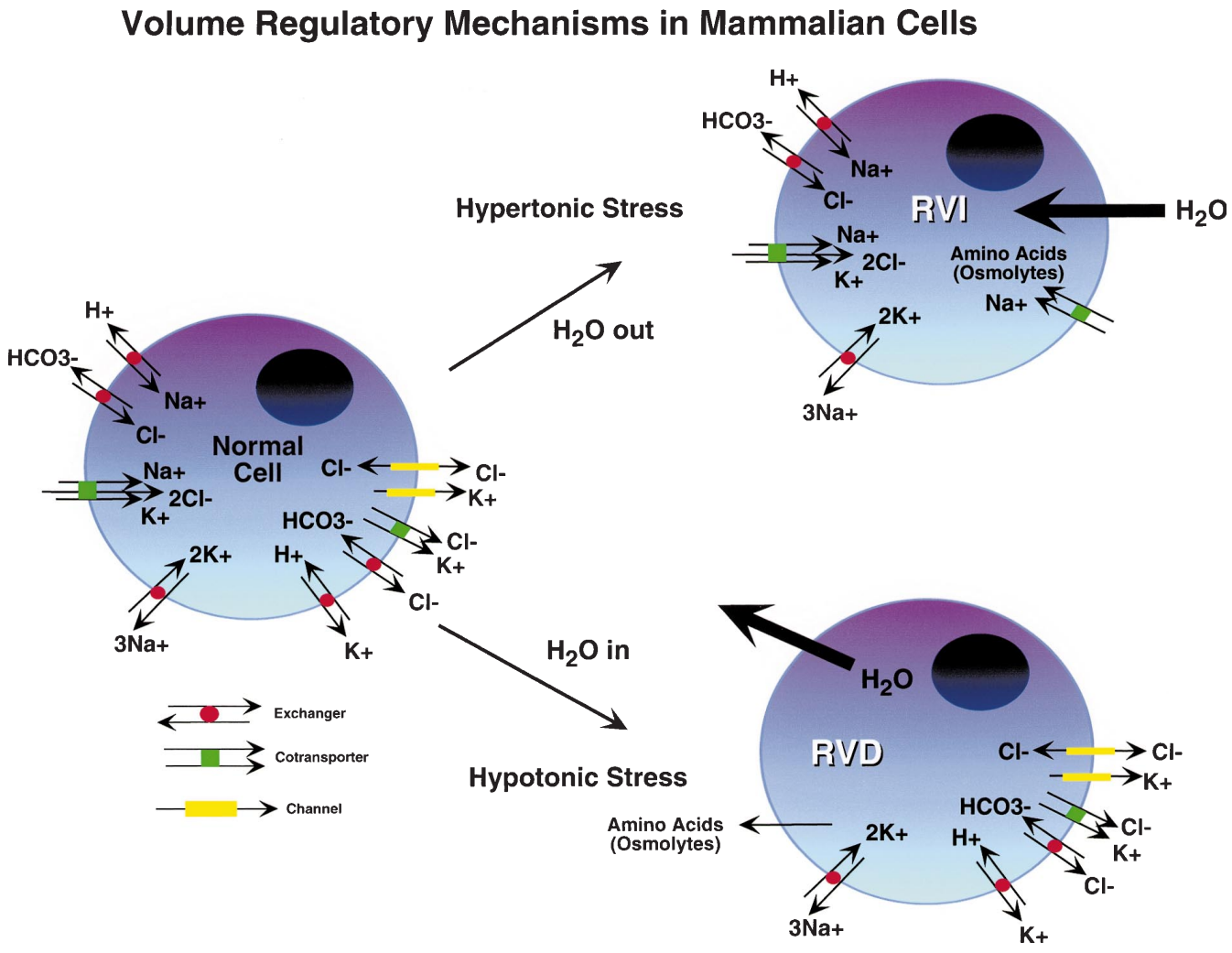

Fig. 1 Volume regulatory mechanisms in mammalian cells. Cells maintain cell volume through the modulation of a repertoire of plasma membrane transporters and ion channels. When cells are exposed to hypertonic stress, they act like osmometers and shrink due to a quick loss of water. Cell volume recovery is triggered by activation of an RVI response. This response includes induction of the $Na^+/K^+/2Cl^-$ cotransporter and the Na^+/H^+ antiporter coupled to the Cl^-/HCO_3^- exchanger. Additionally, Na^+-dependent osmolyte (i.e., amino acids) transporters can be activated and lead to an increase in non-perturbing osmolytes into the cell. During the RVI response, the Na^+, K^+-ATPase is activated and contributes to the extrusion of Na^+ and replacement of Na^+ for K^+. In contrast, when cells are exposed to hypotonic conditions, they swell due to an increase in water content, which activates an RVD response. RVD mechanisms trigger the loss of K^+, Cl^- and obliged water through activation of the K^+/H^+ exchanger coupled to the Cl^-/HCO_3^- exchanger, activation of the K^+/Cl^- cotransporter or via activation of K^+ and Cl^- channels. Loss of intracellular amino acids may also participate in this RVD response (see text)

cellular osmolarity. Therefore, many cells have the potential to sense and quickly respond to changes in the osmotic pressure by activating subsequent volume regulatory mechanisms termed regulatory volume increase (RVI) and regulatory volume decrease (RVD). Both RVI and RVD involve short-term and long-term changes of ion fluxes, osmolyte transport, and changes in gene expression. The primary ions involved in these processes are K^+, Na^+, Cl^-, H^+ and $HClO^-_3$, whereas the most common osmolytes include amino acids, methylamines, polyols, sugars and urea (O'Neill 1999; Perlman and Goldstein 1999).

Ion channels

The plasma membrane is highly permeable to water, which is the major determinant of cell volume. The movement of water across the membrane is largely dictated by the intracellular content of ions and osmotically active substrates that include amino acids and sugars. Due to this permeability of the plasma membrane to water, cells exposed to hypertonic media rapidly shrink, mimicking the response of biological osmometers. However, the existence of RVI mechanisms permits cells to recover their steady-state volume despite a permanent hypertonic threat (Kregenow 1981; Macknight 1988). In general, volume recovery occurs mainly through activation of electroneutral transporters such as the Na^+/H^+ antiporter and the $Na^+/K^+/2Cl^-$ cotransporter (Fig. 1), pos-

sibly by reversible changes in phosphorylation (O'Neill 1999). The antiporter couples its activity with the Cl^-/HCO_3 exchanger and produces a net influx of NaCl and water. To counter the increase in intracellular Na^+, the constitutively active Na^+, K^+-ATPase exchanges Na^+ for K^+ at a much higher rate, and thus provides an electrogenic gradient by maintaining low intracellular Na^+ and, secondarily, a low Cl^- concentration (Grinstein et al. 1982, 1984; O'Neill 1999).

Despite the existence of compensatory mechanisms to counteract hypertonicity, lymphoid cells rarely display RVI and do not recover their volume after hypertonic shrinkage (Bortner and Cidlowski 1996; Hempling et al. 1977). However, Grinstein and coworkers elegantly showed the existence of fully functional RVI mechanisms in lymphoid cells using lymphoblasts and peripheral blood lymphocytes (Grinstein et al. 1984). When exposed to a hypotonic environment, both lymphoblasts and peripheral blood lymphocytes swell, activate RVD mechanisms, and recover their cell volume. Interestingly, when these cells are then placed back in an isotonic medium, they rapidly lose cell volume (isosmotic cell shrinkage), but progressively recover to a near normal cell size through the activation of the RVI response, with a concomitant increase in net intracellular Na^+ and K^+ content and therefore water.

Cells exposed to diluted or hypotonic environments tend to swell and rapidly activate RVD mechanisms (Fig. 1). In many instances, RVD has been found to be coupled to a loss of intracellular K^+, Cl^- and to a lesser extent Na^+, with a concomitant movement of water out of the cell (Cahalan et al. 1987; Strange et al. 1996). For instance, K^+ and Cl^- efflux has been shown to occur in human peripheral blood lymphocytes upon hypotonic shock using isotopic ^{86}Rb and ^{36}Cl efflux measurements (Deutsch et al. 1982; Roti Roti and Rothstein 1973). The extrusion of K^+ and Cl^- along with the obligatory loss of water leads to a reduction in cell volume. However, since K^+ comprises a large fraction of the intracellular osmotic content and a high external K^+ concentration has been shown to inhibit RVD, K^+ efflux seems to play a major role in the RVD responses. Cl^- conductance activated by cell swelling appears to occur to a lesser degree as a result of the loss of K^+, and contributes to a plasma membrane depolarization. These observations on the mechanisms of volume recovery are of particular interest in light of recent studies on lymphocyte apoptosis, in which the loss of K^+ appears to be a major component during the loss of cell volume.

Volume-regulatory plasma membrane transporters

Along with the movement of ions across the plasma membrane, other volume-regulatory mechanisms in mammals include the movement of non-perturbing small organic solutes (osmolytes) through specific plasma membrane transporters, which can be activated by changes in extracellular osmolarity. These non-perturbing or inert small organic solutes (uncharged molecules) protect cells from the deleterious effects of elevated intracellular concentrations of electrolytes (Burg 1995; McGivan and Pastor-Anglada 1994), which may provoke irreversible changes in cellular physiology. This mechanism is crucial in the renal medulla, where physiological situations of hyperosmolarity often occur during the urinary concentrating process. Physiological osmolytes include α-, β-, and γ-amino acids, betaine (a trimethylamine), and polyols (like sorbitol and inositol). Interestingly, these mechanisms are present in cells which are highly unlikely to be exposed to osmotic perturbances (isosmotic existence), suggesting an inherent ability of cells to protect themselves from cell death. Preserving such volume regulatory mechanisms in mammals may have led to an evolutionary advantage in a variety of cellular processes that require altering cell volume changes including apoptosis, proliferation, hypertrophy, atrophy, and differentiation (Bussolati et al. 1996; O'Neill 1999). Following hyperosmotic stress, shrunken cells can activate the uptake of osmolytes as an RVI response to trigger water uptake and volume recovery. In contrast, cell-swelling caused by hypotonicity may lead to a loss of osmolytes and a concomitant loss of intracellular water (Roy and Sauve 1987; Sanchez Olea et al. 1991). For example, human lymphocytes release taurine in response to hypotonic stress and as part of the RVD response (Pasantes-Morales et al. 1991).

Cell volume changes during apoptosis

An early event in cells undergoing apoptosis is the loss of cell volume or cell shrinkage. In what has become one of the most extensively studied model systems for apoptosis, primary thymocytes exhibit profound cell shrinkage following exposure to glucocorticoids (Compton et al. 1987; Wyllie 1980). Thymocytes undergoing programmed cell death display classical biochemical and morphological features of apoptosis including cell shrinkage, chromatin condensation, caspase activation and activity, internucleosomal DNA cleavage, and blebbing of the plasma membrane. Additionally, lymphoid cells can undergo apoptosis in response to a variety of stimuli including T-cell receptor stimulation (Smith et al. 1989), ionizing radiation (Klassen et al. 1993), Fas/CD95 receptor ligation (Matiba et al. 1997; Nagata 1994), chemotherapeutic drugs (Friesen et al. 1996) and Ca^{2+} influx (Caron-Leslie and Cidlowski 1991). One of the most intriguing questions relating apoptotic cell shrinkage to volume regulation is how the cell death pathway is coupled to the activation of cell shrinkage, and whether this cellular shrinkage utilizes cell volume regulatory paths commonly employed in osmotic responses. Unfortunately, the putative "cellular sensors" that specifically dictate the initiation of cell shrinkage during apoptosis are essentially unknown. In the next two sections, glucocorticoid-induced apoptosis and Fas-receptor-mediated apoptosis will be discussed with par-

ticular focus on key aspects of cell volume regulation during the cell death process.

Glucocorticoid-induced apoptosis

Primary isolated rat thymocytes have provided a useful model for the study of the regulation of apoptosis in vivo and in vitro. Glucocorticoid administration to adrenalectomized rats leads to thymic involution and a 50% decrease in the thymocyte population due to apoptosis (Compton et al. 1987). This dramatic cell death response is experimentally reproducible in vitro by treating isolated rat thymocytes with the synthetic glucocorticoid, dexamethasone. In the presence of dexamethasone, freshly isolated rat thymocytes display a rapid dose-dependent loss of cell volume when measured by electronic cell counting (Thomas and Bell 1981). The loss in cell volume was also paralleled by an increase in nuclear fragility of these cells. Interestingly, Klassen and coworkers described a possible two-stage process of apoptotic cell shrinkage in rat thymocytes, the first stage being a sudden and quick loss of cell volume followed by a more gradual loss of cell volume which occurred over an extended period of time (Klassen et al. 1993). Indeed, both stages of cell shrinkage were attributed to a loss of both ions and water. A similar biphasic loss of cell volume has been demonstrated in the human lymphoid cell line CEM-C7 treated with dexamethasone. However, in contrast to the conclusions drawn by Klassen et al., Benson and coworkers suggested in their model that the first stage of cell shrinkage was a result of a loss of water, ions, and cytoplasmic molecules, while the second stage was a result of extrusion of mainly degraded macromolecules (Benson et al. 1996).

Studies from our laboratory using thymic lymphoid cell lines identified a relationship between osmotic-cell volume regulatory mechanisms and apoptotic cell volume regulation (Bortner and Cidlowski 1996). Lymphoid cells were chosen in this study due to their inability to counteract hypertonic stress through RVI responses. S49 Neo cells (an immature murine T-cell line) treated with dexamethasone underwent apoptosis, and displayed all the classical features of programmed cell death. Flow cytometric analysis of dexamethasone-treated S49 Neo cells showed two distinct populations of cells on a forward-scatter (a measure of cell size) versus side-scatter (a measure of cellular granularity) dot plot. One population had a normal cell size, similar to control cells, while a second population had a smaller or shrunken cell size. Further analysis of the DNA integrity of these cells revealed a sub-G1 peak of DNA, indicative of DNA degradation, but only in the shrunken subpopulation of apoptotic cells (Fig. 2). In additional studies, treatment of CEM-C7 cells and primary rat thymocytes with dexamethasone, and Jurkat cells with an anti-Fas antibody to induce cell death, resulted in a similar relationship between cell shrinkage and DNA fragmentation; only the shrunken population of cells had degraded DNA (Fig. 3).

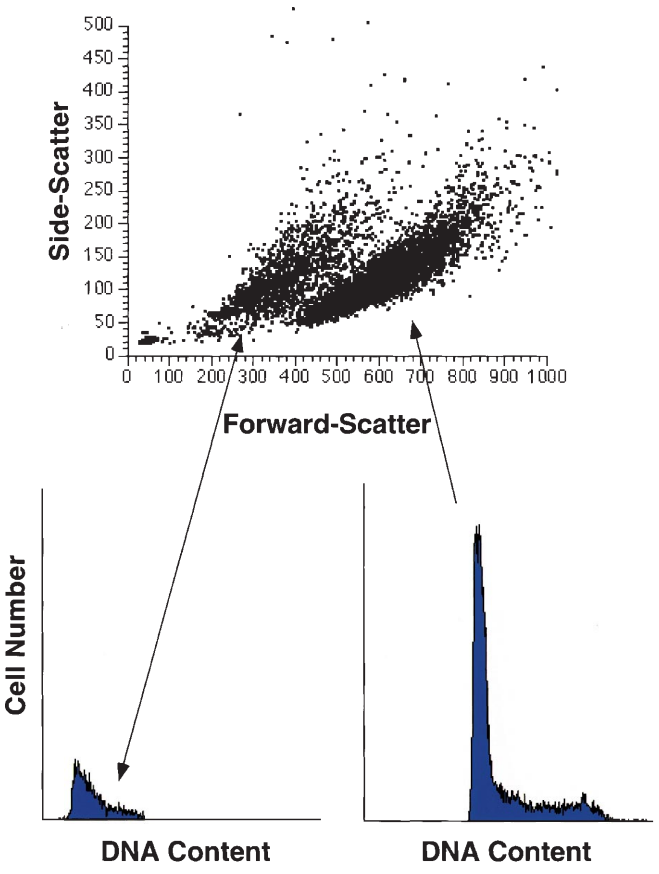

Fig. 2 Relationship between cell size and DNA degradation in dexamethasone-treated S49 Neo cells. S49 Neo cells were treated with 10^{-7} M dexamethasone for 48 h, harvested, then fixed in 70% EtOH overnight at 4°C. Prior to flow cytometric examination, the fixed cells were washed once in 1× PBS and stained in 1 ml 20 µg/ml propidium iodide, 1 mg/ml RNase in 1× PBS. Stained cells were examined on a Becton Dickinson FACSort using CellQuest software. Individual cells (7500) were initially examined on an area vs width dot plot to exclude cell debris and cell aggregates. These cells were then analyzed on a forward-scatter versus side-scatter dot plot to examine changes in cell size and cell granularity, respectively. Two distinct populations of cells were observed on this plot; one population had a high forward-scatter and were of a larger cell size, while a second population had a lower forward-scatter and were of a smaller or shrunken cell size. When each of these populations of cells were individually analyzed for changes in their DNA content, only the shrunken, apoptotic population of cells had degraded DNA. The normal size population of cells had a normal cell cycle histogram [reprinted with permission from Bortner CD, Cidlowski JA (1996) Am J Physiol 271 (Cell Physiol 40):C950–C961; copyright 1996]

Interestingly, S49 Neo cells exposed to a hypertonic environment also underwent cell death, similar to that observed with dexamethasone. In contrast to lymphoid cells, other cell types which have RVI mechanisms to affront anisotonic conditions did not undergo apoptosis upon hypertonic stress. Although the first step of cell shrinkage during hyperosmotic stress is a rapid loss of intracellular water, the second step appears to be a direct induction of the apoptotic cascade primed by the inability of cells to regain their cell volume. Therefore, even though cell shrinkage appears to be a universal hallmark

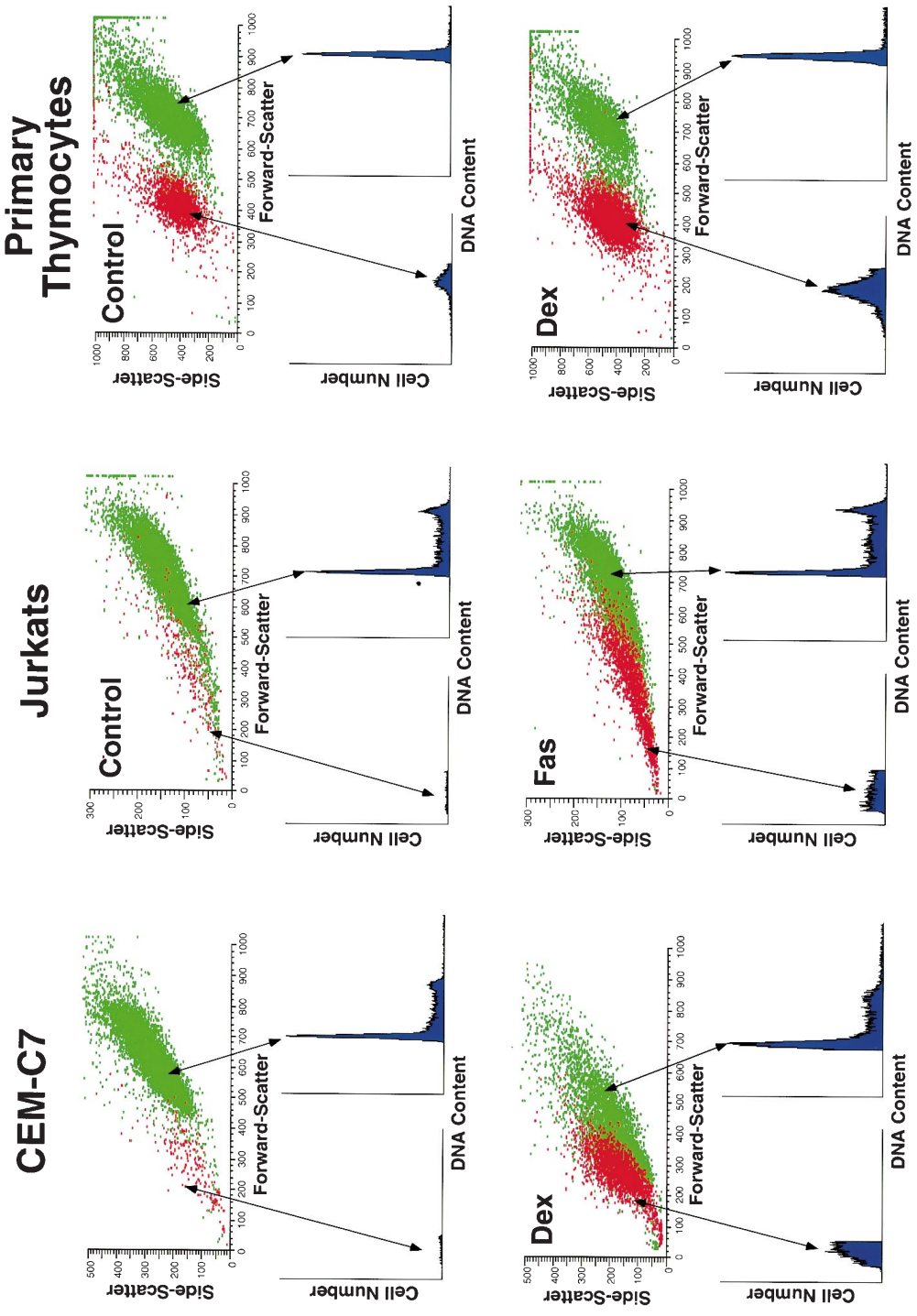

Fig. 3 Relationship between cell size and DNA degradation in various lymphoid cell types. CEM-C7 cells were treated with 2.5×10^{-7} M dexamethasone for 48 h, Jurkat cells were treated with 10 ng/ml anti-Fas antibody for 6 h, and primary rat thymocytes were treated with 1×10^{-7} M dexamethasone for 4 h to induce apoptosis. After the appropriate time, the cells were harvested, fixed in 70% EtOH, and stored at 4°C. Prior to flow cytometric examination, the fixed cells were stained with propidium iodide and examined by flow cytometry as described in Fig. 2. In the presence of each apoptotic stimulus, an increase in the shrunken population of cells was observed compared to the control cells on a forward-scatter versus side-scatter dot plot. Individual analysis of each population of cells for their DNA content showed that only the shrunken population of cells had degraded DNA, while the normal-sized population of cells had a normal cell cycle histogram

of apoptosis, the task of identifying volume regulatory mechanisms during programmed cell death seems to be complicated by the existence of putative specific regulatory signaling molecules and cascades that might be cell type/apoptotic stimulus-dependent.

One of the mechanisms proposed to explain apoptotic cell shrinkage in lymphocytes is a rise in intracellular Ca^{2+} that often occurs early in apoptosis, with subsequent activation of Ca^{2+}-sensitive K^+ or Cl^- channels (Oshimi and Miyazaki 1995). Opening of these channels would consequently force the extrusion of water from the cell. The addition of K^+ channel blockers, such as 4-aminopyridine, sparteine or quinidine, to cytokine-deprived human leukocytes resulted in inhibition of apoptotic cell shrinkage, suggesting the involvement of K^+ efflux as a critical component of the cell death process (Barbiero et al. 1995; Beauvais et al. 1995; Benson et al. 1996). The conclusions from these studies are supported by earlier work where treatment of thymocytes with the K^+ ionophore valinomycin resulted in cell death

(Deckers et al. 1993; Duke et al. 1994). Interestingly, a loss of intracellular Na^+ has also been shown to occur in the shrunken population of cells (Bortner et al. 1997). Recently, our laboratory has shown the importance of K^+ and Na^+ efflux in not only the loss of cell volume, but also the activation of death effectors during apoptosis (Bortner et al. 1997; Hughes et al. 1997). Therefore, it seems clear that changes in the movement of ions across the plasma membrane can actively participate in several different apoptotic events. However, the signals involved in this apoptotic activation of ionic movements are still largely unknown.

The model of Fas-receptor-induced apoptosis

Fas/Apo1 (CD95), a 45-kDa transmembrane protein belonging to the tumor necrosis factor (TNF) receptor superfamily, plays a crucial role in the regulation of the immune system. The induction of programmed cell death by ligation of Fas ligand to its receptor is a model apoptotic system that has been extensively used to dissect the apoptotic cascade (for review, see Ashkenazi and Dixit 1998; Baker and Reddy 1998; Wallach et al. 1999). The importance of this receptor in vivo is evident by an overgrowth of lymphoid organs in conditions where a dysfunction related to the Fas receptor exists (Suda and Nagata 1997). The Fas receptor comprises an extracellular domain (ligand-binding) and a cytoplasmic (effector) domain. During Fas-induced apoptosis, the cytoplasmic region interacts with the death domain of FADD/MORT-1, a cytoplasmic death-domain-containing adapter protein. This recruitment results in the oligomerization, cleavage, and activation of caspase-8, subsequently initiating the caspase cascade.

Studies done in our laboratory revealed the presence of two discrete populations of cells when Fas-receptor-stimulated Jurkat T-cells were analyzed for changes in cell size by flow cytometry. One population had a normal cell size, while a second population had a smaller or shrunken cell size (Bortner et al. 1997). Similar to dexamethasone-induced apoptosis in thymocytes, Fas-induced apoptosis in Jurkat cells resulted in a dramatic decrease in intracellular K^+ in the shrunken population of cells. In addition, effector caspase activity and DNA fragmentation were also restricted to the shrunken, apoptotic cells. The importance of this loss of intracellular K^+ was shown in a series of experiments in which inhibition of K^+ efflux prevented the activation of the cell death program. These results suggest that a sequential activation of the apoptotic machinery occurs after an initial loss of K^+ and cell shrinkage in the Fas pathway.

Although the link between Fas receptor activation and the loss of K^+ is not completely understood, several studies have suggested how movement of intracellular ions may contribute to cell death in the Fas receptor pathway. Recently, several studies have described the inactivation of the *n*-type voltage-dependent K^+ channel through tyrosine phosphorylation during Fas- (Szabo et al. 1996) and ceramide-induced (Gulbins et al. 1997a) apoptosis in Jurkat cells. *N*-type K^+ channels are formed by homotetramers of Kv1.3 subunits, can be activated at potentials more positive than –40 mV, and actively participate in establishing the resting plasma membrane potential in lymphocytes (Cahalan and Chandy 1997; Dupuis et al. 1989). Therefore, inhibition of *n*-type K^+ channels may trigger plasma membrane depolarization during apoptosis. Inactivation of this channel during apoptosis may also contribute to an impairment of K^+ recovery and, thus, secondarily participate in the net loss of K^+ and cell volume during apoptosis. Interestingly, T-cell activation produces the opposing response: induction of Kv1.3 channel expression, and plasma membrane hyperpolarization can be efficiently suppressed by inhibition of the Kv1.3 channel (Cahalan et al. 1987; Koo et al. 1997). In contrast to these results, Gulbins et al. (1997b) were able to inhibit Fas-induced apoptosis using a moderate osmotic stress in Jurkat T-cells, suggesting that the mechanism involved was impaired production of cellular O_2^- that occurs upon ligation of the Fas receptor.

Other ions and osmolytes have also been shown to play an important role in Fas-induced apoptosis. For example, stimulation of the Fas receptor in Jurkat T-cells has been shown to induce activation of the outwardly rectifying chloride channel (ORCC) via a mechanism that involves Src-like kinase-dependent phosphorylation (Szabo et al. 1998). Interestingly, this channel is also involved in the RVD response after hyposmotic stress (Lepple-Wienhues et al. 1998). Fas-receptor-induced apoptosis has also been shown to involve the release of osmolytes such as taurine, which may in part contribute to the loss of cell volume during cell death (Lang et al. 1998a). In the same report, the authors correlated the regulation of taurine efflux with the regulation of DNA degradation, but remained cautious about a putative direct connection between these two processes. Additionally, other osmolytes have also been shown to be released from lymphocytes during the RVD response (Pasantes-Morales et al. 1991), again providing evidence that apoptotic cell shrinkage and the RVD response may be linked during the cell death process. The opposing mechanisms proposed for the modulation of the *n*-type K^+ channels (apoptosis *versus* mitogenesis) raise interesting questions as to how T-cell activation and apoptosis counterbalance one another. It has been shown that cell cycle progression is dependent on an increase in cell volume prior to cell division (Bussolati et al. 1996), which may also influence or interfere with cell volume regulatory mechanisms, and *vice versa* (Perlman and Goldstein 1999). Despite intense research efforts using the well-defined Fas pathway in Jurkat cells, "the potassium channel(s)" responsible for the loss of K^+ during apoptosis has not yet been identified.

Proteases, nucleases and cell shrinkage

Activation of a family of caspases (cysteine-dependent aspartate-directed proteases) during apoptosis initiates

the cleavage of a subset of cellular proteins and drives the process of cell death to a point of no return. Although caspases will not be fully discussed in the present review (for recent literature on caspases, see Nicholson 1999; Slee et al. 1999; Stennicke and Salvesen 1999), an understanding of their sequential and hierarchical mode of action is essential since stages in the apoptotic cascade are often classified as "pre" or "post"caspase activation. The evolutionary importance of caspases in apoptosis has been largely emphasized by the cloning of these proteases in a wide variety of organisms, from the nematode *C. elegans* to *Drosophila* and mammals (Abrams 1999; Horvitz 1999). Caspases are synthesized as proenzymes which are cleaved into mature forms that create an active tetramer. Some members of the caspase family have been shown to act upstream in the signaling of apoptotic cascade (i.e., caspase-8 and -10), whereas others have been typically found to act downstream (i.e., caspase-3 or -9) in the cascade. However, these downstream, or effector, caspases are thought to be directed by upstream, or initiator, caspases. As a consequence of caspase activation, apoptotic nucleases are activated to degrade the chromatin within internucleosomal regions generating the characteristic "DNA ladder" that can be visualized on agarose gels (Cidlowski et al. 1996). This apoptotic nuclease activity irreversibly destroys the genome of the cell. The relationship between caspase and apoptotic nuclease activation and cell shrinkage has recently become an area of active investigation.

A series of studies in our laboratory (Bortner et al. 1997; Hughes et al. 1997) demonstrated a critical and essential role for K^+ loss in both caspase activation and apoptotic nuclease activity. The role of K^+ was analyzed in vitro by examining the loss of this intracellular ion in relation to cell shrinkage, caspase activation, and nuclease activity in extracts from dexamethasone-treated rat thymocytes and anti-Fas-antibody-treated Jurkat cells. Results from these investigations showed that caspase activation in vitro and in vivo were inhibited in the presence of physiological concentrations of K^+ (140–150 mM), while increasing concentrations of KCl (ranging from 0 to 200 mM) suppressed DNA degradation in a dose-dependent manner (Hughes et al. 1997). Furthermore, an apoptotic nuclease activity was completely inhibited at the normal physiological concentration of K^+ (150 mM) (Montague et al. 1997). In a different model, inhibition of K^+ channels with 4-aminopyridine was shown to prevent UV-induced apoptosis in myeloblastic leukemia cells, but failed to prevent etoposide-induced apoptosis in the same cell type (Wang et al. 1999). Therefore, the mechanism of K^+ loss during apoptosis appears to be dependent on the cell type and the apoptotic agent used, compromising the hypothesis that a single channel is involved in the loss of K^+ during apoptosis.

The relevance of the K^+-concentration-dependent regulation of key apoptotic enzymes during apoptosis in thymocytes was also demonstrated by physically sorting apoptotic cells into normal and shrunken subpopulations by flow cytometry. These experiments showed that caspase activation and nuclease activity were restricted to shrunken cells with low potassium content (Hughes et al. 1997). The involvement of caspases in glucocorticoid-induced cell shrinkage of thymocytes was also investigated using a general non-competitive inhibitor of caspases, zVAD. In this study, both cell shrinkage and chromatin degradation were prevented by caspase inhibition, suggesting that activation of initiator caspases may precede the loss of cell volume and activation of nucleases (Hughes and Cidlowski 1998).

The requirement for K^+ loss (with the subsequent cell shrinkage) prior to apoptotic nuclease activation during cell death has also been shown in dexamethasone-treated S49 Neo cells (Bortner et al. 1997). In this study, only the shrunken apoptotic cells with a low intracellular K^+ concentration had caspase activity and degraded DNA. In addition, Walev et al. (1995) demonstrated that K^+ depletion in monocytes resulted in activation of caspases. Interestingly, distinct signal transduction pathways may also be important in determining the caspase-dependent/independent occurrence of various apoptotic characteristics. In a recent study in our laboratory (Bortner and Cidlowski 1999), treatment of Jurkat T-cells with either an anti-Fas antibody, the calcium ionophore A23187, or the calcium ATPase inhibitor thapsigargin resulted in each case in the classical characteristics of apoptosis including cell shrinkage, loss of intracellular K^+, loss of the mitochondrial membrane potential, and DNA degradation. Anti-Fas-induced apoptosis in Jurkat cells was completely prevented by the pan-caspase inhibitor zVAD, while cells treated with either A23187 or thapsigargin in the presence of zVAD were only partially prevented from undergoing apoptosis, as determined by the loss of cell volume, intracellular K^+, and mitochondrial membrane potential. These data suggest the presence of a caspase-independent signaling pathway of programmed cell death, depending on the particular stimulus employed. Additionally, inhibitors of either caspase-3 (DEVD) or caspase-8 (IETD) only partially prevented anti-Fas-induced cell death, while being almost totally ineffective in preventing either A23187- or thapsigargin-induced apoptosis. Changes in ion fluxes, cell volume, and activation of caspases preceded the onset of nuclear events that occur during apoptosis. The extent of degraded DNA with each apoptotic stimulus in the presence of the various caspase inhibitors was always completely prevented, suggesting that cell shrinkage, K^+ efflux, and changes in the mitochondrial membrane potential are tightly coupled, but can occur independently of DNA degradation.

Additional aspects of cell volume regulation

Although current information describing the signaling mechanisms involved in apoptotic cell shrinkage has focused on events unique to the cell death process, signaling of osmotic processes (Haussinger and Schliess 1999) as well as changes in the cytoskeletal network should not

be ignored. Hyperosmotic and hyposmotic stress induce changes in the phosphorylation status of a subset of different proteins. Both types of osmotic stress induce phosphorylation of MAPKs (mitogen-activated protein kinases), which could be a consequence of activation of stress responses. Cellular MAPKs, such as Erk-1 and -2, are activated in response to hypotonicity, but, depending on the cell type, different signaling pathways are involved in this modulation. JNKs (c-*jun* kinases, alternatively known as stress-activated protein kinases, SAPKs) are activated upon hypertonicity (Galcheva-Gargova et al. 1994). Protein kinase C (PKC) has also been shown to be involved in cell-volume regulatory process in a variety of cell types (Grinstein et al. 1986; Larsen et al. 1994). In addition, different tyrosine kinases are activated during RVI responses in neutrophils (Krump et al. 1997). Many signaling mechanisms, depending on the apoptotic pathway and the cell type considered, seem to be involved in the apoptotic cascade. For example, Fas receptor stimulation induces activation of JNK/SAPK (Latinis and Koretzky 1996; Wilson et al. 1996). Furthermore, MAPK (Holmstrom et al. 1998; Yeh et al. 1998) and PKC activation (Ruiz-Ruiz et al. 1997) can protect cells from Fas-induced apoptosis. Also of interest is a serine/threonine protein kinase known as *sgk* (Webster et al. 1993), which is induced during serum starvation, glucocorticoid treatment, and hyperosmotic stress (Waldegger et al. 1998). However, the substrate for this kinase is unknown and regulation of apoptotic cell shrinkage by various kinases remains an unexplored area. Therefore, a variety of signaling processes could be considered as candidates to regulate cell shrinkage during apoptosis.

The cytoskeleton also deserves some attention in relation to the loss of cell volume during apoptosis. Changes in cell volume are accompanied by reorganization of cytoskeleton and associated proteins, which are also involved in sensing and mediating changes in cell volume (Moustakas et al. 1998). Interestingly, the cytoskeleton-associated protein fodrin has been shown to be cleaved by a caspase-dependent mechanism during apoptosis (Martin et al. 1995). Fodrin is also important in anchoring a variety of proteins to the plasma membrane, including ion channels and transporters [i.e., the Na,K-ATPase (Nelson and Hammerton 1989)], which suggests that fodrin may play an important role during late (postcaspase) stages of cell shrinkage during apoptosis, an hypothesis that has not been explored.

Summary

Loss of intracellular K^+ and cell shrinkage during an early phase of apoptosis appear to be obligatory events for the activation of death enzymes (caspases, endonucleases) and, therefore, critical for the progression of the death program. Although several mechanisms have been suggested as mediators of cell shrinkage during apoptosis, the precise signaling events at the plasma membrane that "switch on" such mechanisms during apoptosis are unknown. Although K^+ movement across the plasma membrane plays a pivotal role in apoptosis, the channel(s) responsible for K^+ efflux has not been defined despite many attempts to do so. These unfruitful efforts might be in part due to the involvement of several ion channels/transporters that operate in a coupled or a "more complex than expected" regulatory fashion during apoptotic cell shrinkage. An extensive amount of experimental data has shown a primary role for ion fluxes and cell shrinkage during apoptosis (see, for example, Bortner and Cidlowski 1998; Lang et al. 1998b). However, little is known about the precise control of ion channels, transporters, and signaling pathways responsible for cell shrinkage during apoptosis. Undoubtedly, the discovery of new properties of ion channels and associated proteins will shed light on the mechanisms responsible for the loss of cell volume during apoptosis.

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