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APOPTOSIS AND CELL VOLUME REGULATION

The importance of ions and ion channels

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1. INTRODUCTION

The shrinkage of cells is one of the earliest recognized morphological characteristics of apoptosis or programmed cell death. Even though the mechanisms underlying volume regulation of cells as a response to anisotonic conditions have been intensively studied for decades, the mechanisms, ion channels and transporters involved in cell shrinkage during apoptosis are far less understood. However, it is clear that the movement of ions and the resulting change in the ionic intracellular environment are important components of programmed cell death. They are ultimately involved in both the activation of the biochemical signaling cascades and the volume changes that occur during apoptosis. In this article, we review the general mechanisms of volume regulation and the distinct differences to the cell volume decrease associated with apoptosis. We also address the role of certain ions and how they are involved in the regulation of the signaling cascades.

2. APOPTOSIS – PROGRAMMED CELL DEATH

2.1 Types of Cell Death

The number of cells in multicellular organisms often remains constant throughout life and is dictated by a tightly regulated balance between cellular differentiation, cell proliferation and also cell death. The ability to remove unwanted cells from tissue and organs is crucial for the normal homeostatic function of an organism. Based on their different morphological and biochemical characteristics**,** two types of cell death can be readily distinguished.

Necrosis is considered to be a pathological or accidental form of cell death. It is characterized by massive cell swelling, destruction of intracellular organelles and finally

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a rupture of the plasma membrane.[1] Subsequently, the cellular content is liberated into the surrounding tissue resulting in an inflammatory response from adjacent cells. The observed increase in cell volume occurs in both the cytoplasm and cellular organelles, while the volume of the nucleus is largely unchanged. Cellular DNA, RNA and proteins are randomly degraded and DNA appears as a smear when examined by agarose gel electrophoresis. Necrosis is usually caused by severe forms of damage or by the loss of a positive energy balance of a cell.[2] Under this condition, impaired ATP dependent ion pumps are unable to preserve the intracellular ion balance, resulting in an influx of water into the cell causing massive cellular swelling and finally rupture of the cell membrane.

In contrast, apoptosis is characterized by cell shrinkage, selective degradation of proteins, condensation of the nuclear chromatin and a characteristic fragmentation of the DNA.[1] Cellular shrinkage can be observed in both the cytoplasm and the nucleus of apoptotic cells and is a universal characteristic of the programmed cell death process. During apoptosis, the DNA is specifically cleaved at the linker region between adjacent nucleosomes by endogenous endonucleases. The resulting 180-200 bp DNA fragments appear as a characteristic ladder-like pattern when visualized by agarose gel electrophoresis. Often, plasma membrane surrounded particles, so-called apoptotic bodies, appear which are engulfed by neighboring cells or macrophages.[3] The morphological changes characteristic of apoptosis are based on a highly regulated, conserved endogenous cell death program that effectively removes unwanted cells from the organism in a non-inflammatory manner.[4] Apoptosis is a metabolically active energy-demanding process which maintains plasma membrane integrity and cellular energy levels until late in the cell death process.

Functionally, apoptosis plays a significant role during the embryonic development of an organism. Furthermore, auto-responsive immune cells or infected cells are removed by programmed cell death. Considering the growing number of diseases that are linked to abnormal rates of apoptosis, the importance in understanding the biochemical processes defining programmed cell death becomes obvious. “Excessive” apoptosis can lead to neurodegenerative diseases,[5] rheumatoid arthritis,[6] and AIDS,[7] whereas “insufficient” programmed cell death can lead to inflammation,[8] autoimmunity,[9] tumorigenic growth and cancer.[10]

2.2 The Apoptotic Signaling Cascades

In contrast to the blunt trauma resulting in necrosis, the selective removal of cells by apoptosis is induced by either specific extracellular (extrinsic) or intracellular (intrinsic) stimuli. These stimuli trigger endogenous signaling cascades that activate apoptosis resulting in specific morphological and biochemical cellular changes (Figure 1).

Extrinsically, apoptosis is induced by activating receptors of the tumor necrosis factor family (TNFR-family) by binding of a specific ligand (e.g., FasL) to its corresponding receptor (e.g., FasR) on the extracellular surface of a cell. The resulting aggregation of the receptor leads to the formation of an intracellular complex called death-inducing-signaling-complex (DISC).[11] This complex recruits various adaptor molecules and a zymogene of a special class of proteases known as caspases. Caspases are a family of cysteine-proteases that stand in the center of the apoptotic signaling cascade. Their activation by either dimerization (initiator caspases) or cleavage of the inactive pro-enzyme (effector caspases) is of considerable importance for the apoptotic progression. For example, after the initiator caspase-8 has been activated in the DISC, it further

activates downstream effector caspases like caspase-3, -6 and -7. These effector caspases are responsible for the cleavage of various cellular substrates. Death substrates include endonucleases which once proteolytically activated are responsible for the specific degradation of the DNA as well as kinases, cytoskeletal structures, ion pumps, and regulatory proteins, all of which coordinate the morphological and biochemical changes observed during programmed cell death.

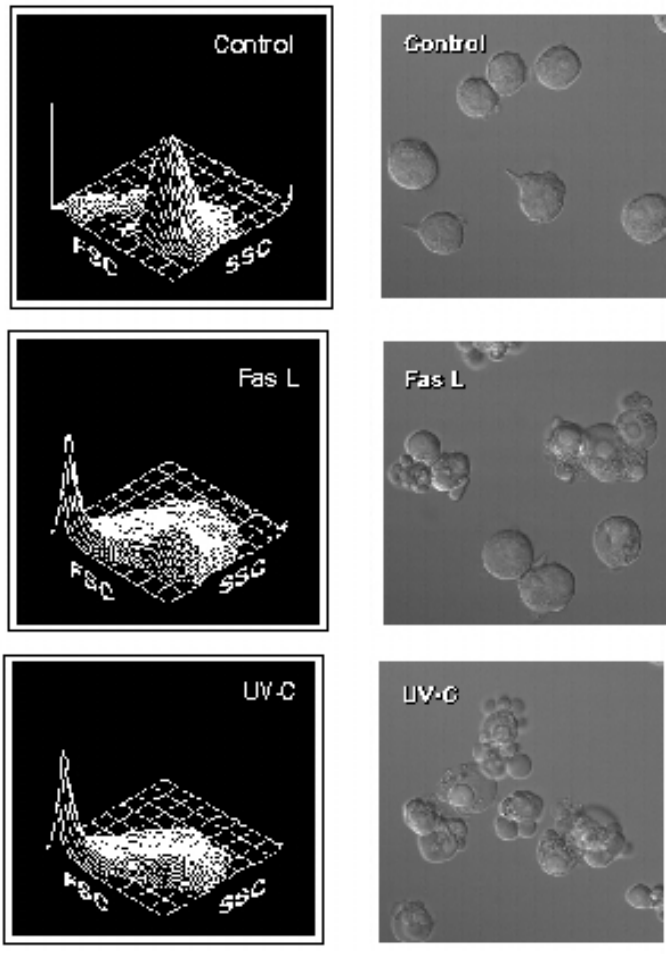

Figure 1. Cell shrinkage and apoptotic body formation occurs during Fas ligand- and UV-induced apoptosis in Jurkat T-cells. Jurkat cells treated in the presence and absence of 50 ng/ml Fas Ligand or 60 mJ/cm^2 UV-C for 4 hours were initially examined for changes in cell size by flow cytometry. Cells were analyzed on a forward-scatter (FSC; cell size) versus side-scatter (SSC; cell granularity) 3D plot. A decrease in FSC indicates a loss in cell size. Morphological examination of Jurkat cells treated with either Fas ligand or UV-C using Differential Interference Contrast (DIC) microscopy showed the classical apoptotic morphology for the Fas ligand and UV-C treated cells.

Apoptosis can also be induced intrinsically by a variety of stimuli including UV-irradiation, various pharmacological stimuli like staurosporine or actinomycin D, removal of growth factors and survival signals as well as intracellular generation of reactive oxygen species (ROS). The hallmarks of the intrinsic, apoptotic, signaling pathway are centered on alterations in the mitochondria. These include changes in the mitochondrial morphology, the mitochondrial membrane potential and other bioenergetic changes depending on the respiratory chain. The mitochondria sequester in the intermembrane space a variety of apoptogenic factors like cytochrome c, apoptosis inducing factor (AIF) and Smac/DIABLO that are released into the cytosol during programmed cell death. However, release of these proteins is often, but not exclusively, regulated by a protein family called the Bcl-2 family. Once the apoptogenic factors are released into the cytosol, they participate in the progression of the apoptotic-signaling cascade. Best characterized is the formation of a high molecular weight complex called the apoptosome consisting of the apoptosis-protease-activating-factor-1 (APAF-1), cytochrome c, ATP and pro-caspase-9. In the apoptosome, the initiator caspase-9 becomes activated which in turn activates effector caspases by proteolytic cleavage, resulting in further degradation/activation of the death substrates.

It should be noted that many of the morphological changes involving proteins and other characteristics of programmed cell death often depend on the apoptotic stimulus and the cell type undergoing cell death. Furthermore, during programmed cell death, not all the characteristics of apoptosis are necessarily observed in every situation. Variation of the actual outcome of certain apoptotic hallmarks can be observed in different cell lines or after triggering programmed cell death with different stimuli suggesting that not all the features of apoptosis are necessary for cell death.

3. VOLUME REGULATION AND APOPTOSIS

In general, mammalian cells maintain a constant shape and cellular volume in the environment of a tissue. However, during cell division, differentiation, migration, and apoptosis, tremendous volume changes can occur in cells as the result of ion movement. The plasma membrane of all cells contains various ion channels and transporters that allow the cell to maintain a homeostatic plasma membrane potential and counteract osmotic imbalances. Additionally, during their life span, cells get exposed to anisotonic conditions in accordance to their tissue location. Therefore, the ability to regulate their cellular volume is crucial for their survival and function.

3.1 Regulatory Volume Increase (RVI)

The plasma membrane of cells is highly permeable to water due to the presence of water permeable channels called aquaporins. By exposing cells to a hyperosmotic condition, an immediate and rapid cell shrinkage occurs.[12] This shrinkage is the result of an efflux of intracellular water brought about by the higher extracellular osmolarity. This "stress-induced shrinkage" activates a cellular response known as regulatory volume increase (RVI). By activating an influx of Na^+ and Cl^-, cells recover their original volume. The net uptake of NaCl takes place through a coupled activation of the Na^+/H^+ antiporter, the $Na^+/K^+/2Cl^-$ cotransporter, and the $Cl^-/HCO3^-$ exchanger. Their activation is probably achieved by reversible changes in phosphorylation.[13] The uptake of organic

osmolytes like amino acids and sugars also contributes to the increase of the intracellular osmolarity during RVI.[14] The co-uptake of these non-perturbing or inert small organic solutes protects the cell from the harmful effects of an elevated electrolyte concentration which may provoke irreversible changes in cellular physiology.[15, 16] The Na^+/K^+-ATPase exchanges this excess of intracellular Na^+ for K^+ and restores normal, homeostatic ionic balance in an energy-dependent manner.

3.2 Regulatory Volume Decrease (RVD)

On the other hand, placing cells into a hypotonic environment results in rapid cell swelling due to an influx of water that triggers a regulatory volume decrease (RVD). The loss of intracellular water is mainly achieved by a massive extrusion of intracellular K^+, Cl^- and to a lesser extent Na^+ ions.[17] K^+ channels, K^+/Cl^- cotransporters, K^+/H^+ and Cl^-/HCO_3^- exchangers and some amino acid transporters participate in this ion efflux. A high external K^+ concentration abolishes the RVD response, providing evidence for the importance of the K^+ loss. The Cl^- conductance activated by cell swelling appears to occur to a lesser degree as a result of the loss of K^+ and contributes to depolarization of the plasma membrane. Both RVI and RVD clearly involve short-term and long-term changes of ion fluxes, osmolyte transport, and changes in gene expression.

Mechanical changes in the plasma membrane, the integrin network, and macromolecular crowding have been postulated to represent potential cellular osmosensors responsible for triggering the volume regulatory mechanisms.[18] Osmosensing and the resulting changes in the cellular volume induce a profound reorganization of the cytoskeleton which in turn might immediately affect the transduction of extracellular signals.[19] In general, cell swelling and cell shrinkage affect the cytoskeletal architecture. Swollen cells are known to contain depolymerized actin filaments, possibly due to Ca^{2+} binding to gelsolin.[20] However, an intact actin filament network is required for Na^+ channel activity, insertion of volume regulatory channels into membranes, regulation of channels by kinases and phospholipids, activation of mechanosensitive anion channels by membrane stretch, and activation of the Na^+/H^+ exchanger as well as the $Na^+/K^+/2Cl^-$ cotransporter.[20]

3.3. Apoptotic Volume Decrease (AVD)

The loss of cell volume or cell shrinkage, termed apoptotic volume decrease (AVD) is a general morphological event associated with apoptosis that clearly differentiates programmed cell death from necrosis. The reason for the loss of cellular volume is still unknown; although, one could hypothesize that engulfment of a dying cell is favored by a decreased cell volume. However, as described in detail below, the changes in ions involved in AVD also play a key role in the actual activation of the programmed cell death signaling cascade. The time frame in which apoptotic cell shrinkage can be detected appears to be cell type-, apoptotic stimulus-dependent and is often stochastic in cultured cells. The loss of cell volume is the result of the combined action of ion channel fluxes, plasma membrane transporter activity, and cytoskeletal reorganization, all leading to a massive extrusion of intracellular water. It is noteworthy that cellular shrinkage during AVD is not counteracted by a regulatory volume increase response (RVI). In fact, cells undergoing AVD after induction of apoptosis appear to react with a facilitated RVD response to application of hypotonic stress.[21] This has lead to the assumption that the

volume-regulatory K^+ and Cl^- channels that participate in AVD are most likely, in part, the same channels activated during RVD response. Furthermore, it is now well-established that the loss of intracellular ions, specifically potassium and sodium are important for AVD.[22] To maintain electroneutrality, the loss of anions like chloride should accompany the loss of sodium and potassium. Additionally, as previously described, a potential involvement of cytoskeleton should not be ruled out as important for sensing and mediating volume changes. It has been shown that the cytoskeleton-associated protein fodrin was cleaved by a caspase-dependent mechanism during apoptosis.[23] Fodrin is also important in anchoring a variety of proteins to the plasma membrane, including ion channels and transporters.[24] This suggests a potential role for fodrin in a late (post-caspase) stage of apoptotic cell shrinkage.

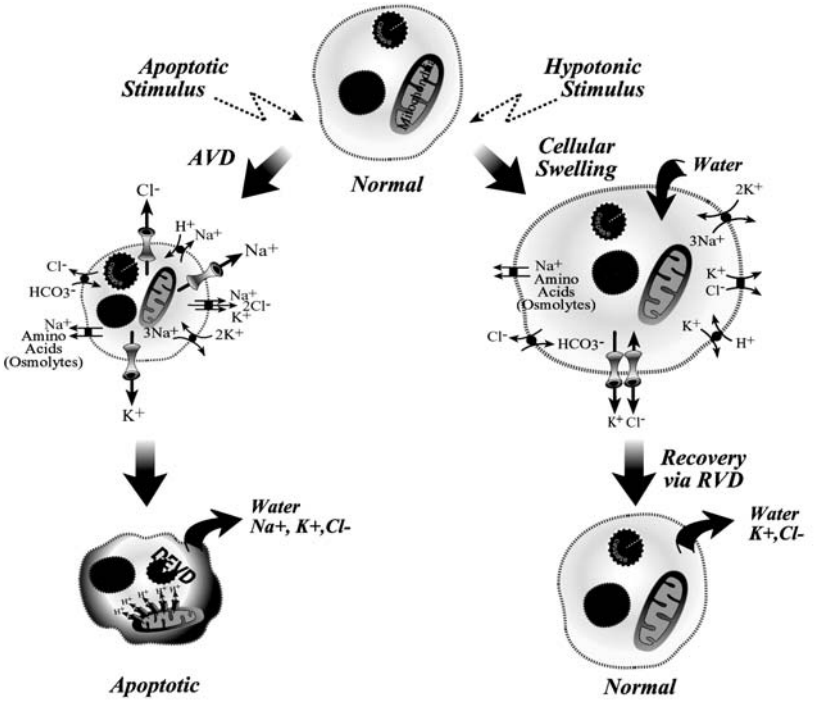

Figure 2. A loss of cell volume occurs during both AVD and RVD; however, AVD occurs in an isotonic environment while RVD occurs as a response to a decrease in extracellular osmolality. Both responses may utilize similar channels and ionic transporters to achieve the overall goal of a reduction in cell size.

It is noteworthy that while sharing similar characteristics, the apoptotic volume decrease (AVD) response is different from a regulatory volume decrease (RVD) response (Figure 2). In both cases, the loss of intracellular water by extrusion of ions and organic osmolytes might involve the same ion channels, exchangers and transporters. However, differences in the activation and signaling processes must exist because RVD appears to

occur exclusively as a response to hypotonic stress, whereas AVD is triggered by multiple and unique stimuli and occurs in an isotonic environment. Additionally, differences in the movement of water may also exist between RVD and AVD. In a study examining water movement during apoptosis, inhibition of aquaporin activity or overexpression of aquaporins was shown to prevent or enhance AVD, respectively.[25] Interestingly, swelling assays showed that the shrunken, apoptotic population of cells had a very low permeability to water compared with the normal non-apoptotic cells, suggesting that aquaporins become inactive after the AVD process. Thus, aquaporin inhibition after AVD may facilitate the occurrence of a low intracellular ionic environment shown to be essential for optimal activation of apoptotic enzymes (see below). It will be a challenging future task to characterize the osmosensors involved in RVD and the key regulators activated during programmed cell death that make use of the same mechanisms to reach the ultimate goal of cellular volume decrease.

4. THE ROLE OF IONS FOR THE ACTIVATION OF APOPTOSIS

The loss of ions and other organic osmolytes appears to be a driving force for the AVD response during the progression of apoptosis. The resulting decrease in ionic strength is not only necessary for the AVD response, but it also plays a pivotal role in the activation of the apoptotic-signaling cascade. In many cases, caspase and nuclease activity could not be detected prior to apoptotic cell shrinkage, suggesting that these classes of enzymes are in some way regulated by changes in cell volume and ionic fluxes.

Using FACS analysis to physically sort cells, it was shown that in Jurkat cells after induction of apoptosis with an anti-Fas antibody only the shrunken cell population contained significantly reduced levels of intracellular K^+ and Na^+.[22] Furthermore, active caspase-3, one of the most important executioner caspases, as well as significant DNA degradation were restricted to the cell population with decreased cellular volume. Culturing Jurkat cells in medium composed of high K^+, thus preventing the loss of K^+, resulted in inhibition of the apoptotic signaling cascade and cell shrinkage.[22] It was also shown that apoptosis was enhanced after decreasing the intracellular K^+ concentration by application of a prior RVD response. Jurkat cells cultured under hypotonic conditions showed an 85% faster apoptotic progression after stimulation with anti-Fas antibody. Interestingly, the decreased K^+ concentration only potentiated the cell death process, because hypotonic conditions alone did not induce programmed cell death. Altogether, this provided the first evidence for a direct link between the apoptotic machinery and changes in the intracellular ionic composition and suggested that the loss of intracellular K^+ is required for the activation of the programmed cell death-signaling cascade.

In vitro studies have also shown that the dATP/cytochrome c-dependent activation of caspase-3 is highly dependent on the K^+ concentration. A physiological K^+ concentration actually inhibited caspase-3 activation, whereas a decrease of the K^+ concentration resulted in dose dependent caspase-3 activation.[26] Furthermore, formation of the apoptosome, a high molecular weight protein complex in which caspase-9 is processed to its active form, is inhibited *in vitro* by increasing K^+ concentrations.[27] Similarly, the activation of interleukin-1ß-converting enzyme (ICE, a synomym for caspase-1) is also directly influenced by K^+ flux and local K^+ concentration.[28] Together, these data suggest that the efficient activation of caspases depends on changes in the intracellular K^+ concentration.

In vitro studies have also shown that nucleases are inhibited by physiological K^+ concentrations.[26] Additionally, substituting the K^+ ions in the buffer for other monovalent ions like Na^+, Cs^+ or Li^+ led to a similar dose-dependent repression of the nuclease activity. This data suggested that the ionic strength and not necessarily K^+ itself is responsible for impairment of nuclease activity. Using isolated nuclei from HeLa cells exposed to buffers with increasing K^+ concentrations, it was directly shown that increasing concentrations of K^+ prevented internucleosomal DNA cleavage, characteristic for apoptosis, in a dose-dependent manner.[29] Furthermore, DNA fragmentation was blocked by physiological K^+ concentrations, indicating that the ionic strength and/or the ionic composition might act as a safeguard against accidental DNA degradation. In the case of caspase activated DNase (CAD), an important nuclease in the execution of apoptosis, the dependence on ionic strength was directly shown with naked DNA *in vitro*.[30]

Together these data on the activation of caspases and the activity of nucleases, both of which share a central role in the progression and execution of apoptosis, provide evidence for the critical prerequisite of a decrease in intracellular ionic strength. Since K^+ is the most prominent intracellular ion, a decrease in the K^+ concentration is pivotal and may act as a regulator of apoptosis triggered by various stimuli. Furthermore, it is also possible homeostatic levels of potassium act as a safeguard against the accidental activation of the enzymes usually engaged to execute the cell death program.

5. THE ROLE OF ION CHANNELS DURING APOPTOSIS

As indicated above, the loss of K^+ appears to be an important ion-related regulator for the activation of at least two different classes of enzymes required for apoptosis as well as the apoptotic volume decrease. However, the involved ion channels, exchangers or transporters as well as the exact communication between the apoptotic trigger and ion channel activation are not well characterized. Because of the obvious analogy between K^+ extrusion during a RVD response and an AVD response, the importance of K^+ channels during apoptosis has been extensively studied. Using K^+ channel blockers like 4-aminopyridine (4-AP), sparteine or quinine, the shrinkage of human eosinophils was abrogated after induction of apoptosis by cytokine withdrawal.[31] Furthermore, 4-aminopyridine prevented UV-C evoked K^+ current as measured by patch-clamp techniques and prevented the loss of viability after UV-C treatment of myeloblastic leukemia cells (ML-1). The UV-C stimulated K^+ channel activity appears upstream of the JNK signaling pathway, and Ca^{2+} influx does not play a significant role in the JNK signaling of UV-C induced apoptosis. Interestingly, 4-AP did not prevent cell death induced by etoposide in the same cell line.[32] Additionally, in a rat liver cell line (HTC), activation of an outwardly rectifying K^+ channel after apoptotic stimulation with TNF-α was shown to be sensitive to Ba^{2+} and quinine (a K^+ channel blocker). In this study, the channel opening was Ca^{2+}, ATP and PKC, but not calmodulin kinase II, dependent.[33] However, in other cell types such as astrocytes, TNF-α has been shown to be a potent inhibitor of inwardly rectifying K^+ currents.[34] In monocytic cells (U937), cell shrinkage was prevented by Ba^{2+} and quinine and various Cl^- channel blockers (NPPB, DIDS and SITS).[21] Altogether, these examples demonstrate the variation in the activation pathway

of apoptosis depending on the stimulus and cell type, thus distinguishing the characteristic ion fluxes of AVD from RVD.

The n-type K^+ channels are an important target for tyrosine phosphorylation. Stimulation of apoptosis in Jurkat T-cells with anti-Fas antibody resulted in a fast inhibition of the voltage-dependent n-type K^+ channel ($K_V1.3$). The current inhibition correlated with tyrosine phosphorylation of the channel protein, indicating a link between induction of apoptosis, tyrosine kinases and n-type K^+ channels.[35] Interestingly, in a second study, acute treatment of human Jurkat T-lymphocytes (E6.1) with anti-Fas antibody resulted in activation of a K^+ current within 30 minutes. This current was blocked by nanomolar concentrations of margatoxin or ShK-Dap selective blockers of $K_V1.3$ channels.[36] The activation of $K_V1.3$ channel mediated K^+ current was caspase-8 dependent and independent of *de novo* protein synthesis. This current appeared before detectable cellular shrinkage, suggesting an early role in the apoptotic signaling cascade upstream of caspase-3. Furthermore, activation of PKC activity known to decrease Fas-induced apoptosis also decreased $K_V1.3$ channel mediated K^+ currents. However, it is noteworthy that blocking the $K_V1.3$ channel mediated K^+ currents with ShK-Dap still resulted in cellular shrinkage after triggering apoptosis by Fas ligation.[36] This implicates other ion channels in the loss of K^+ during apoptosis and highlights the complex nature of ion movement during the cell death process.

Subsequently, it was shown that CTLL-2 T-lymphocytes deficient in $K_V1.3$ are resistant to apoptosis induction by actinomycin D. Transfecting the $K_V1.3$ channel back into these cells restored sensitivity to this apoptotic stimulus.[37] Actinomycin D triggers the release of ROS and induces intracellular acidification. Furthermore, actinomycin D might sensitize cells to Fas ligation by downregulation of FLIP, an inhibitor of caspase-8. Cells deficient in $K_V1.3$ showed no release of cytochrome c from the mitochondria, no loss of the mitochondrial membrane potential, and no DNA fragmentation, indicating an early requirement of this ion channel in the activation phase of apoptosis. However, the activation of $K_V1.3$ mediated K^+ currents appeared late after application of actinomycin D, suggesting a secondary role of $K_V1.3$, independent of its ion channel function.[37] In this scenario, $K_V1.3$ might function as a platform required for assembling of pro-apoptotic proteins.

Interestingly, in thymocytes from $K_V1.3$-deficient mice, no voltage-dependent K^+ current was detected; however, transcripts for other voltage-dependent K^+ channel proteins such as $K_V1.4$ were upregulated. To compensate for this loss of $K_V1.3$, a 50-fold increased Cl^- current was observed in the $K_V1.3^{-/-}$ thymocytes compared to the wild-type cells which could be blocked by NPPB.[38] This data indicates that impaired ion flux through a specific ion channel influences the protein expression and activity of similar or compensatory ion channels. The appearance of a compensatory anion current, in this case of Cl^-, provides an interconnection and importance of ion movement and suggests the movement of more than one ion during programmed cell death. A compensatory ion movement also indicates that once initiated, programmed cell death seals the fate of a cell and dooms it to death. This is important for immune cells which are critical for the overall well-being of a multicellular organism.

It appears that the K^+ channels involved and the signaling pathways that lead to their activation depend on the apoptotic stimulus and the cell type. Furthermore, these studies prove the importance of ion channel inhibitors as modulators for the progression of apoptosis. However, simply inhibiting ion flux *in vivo* is of lesser value in the prevention

of apoptotic cell death due to the diversity of cell types and the multiple stimuli that can induce cell death through numerous pathways in any given organism.

Knowing the importance of K^+ and Cl^- efflux during a RVD response, the possible involvement of Cl^- channels has also been evaluated during apoptosis. It was shown that induction of apoptosis by Fas ligation triggers the activation of an outwardly rectifying chloride channel (ORCC) in Jurkat T-lymphocytes. The activation is mediated by Src-like tyrosine kinase dependent phosphorylation.[39] Furthermore, inhibition of ORCC with glibenclamide or indoleacetic acid (IAA) resulted in decreased intracellular acidification and also decreased apoptotic cell death, indicating that Cl^- efflux is required for apoptotic progression. The occurrence of a separate Cl^- efflux through ORCC during the AVD response suggests that the efflux of K^+ and Cl^- may occur in parallel, similar to a RVD response, through two separate efflux pathways. However their activation must somehow be coordinated during the progression of programmed cell death.

Apoptotic cell death in HeLa, U937, NG108-15 and PC12 cells induced by either staurosporine or TNF/CHX was significantly reduced by a variety of volume-regulatory chloride channel inhibitors including NPPB, SITS, DIDS, niflumic acid, glibenclamide and phloretin.[21] Interestingly, in all cases, application of the volume-regulatory chloride channel inhibitors prevented cell shrinkage, release of cytochrome c from mitochondria, effector caspase activation and DNA laddering. In contrast, blockers of cAMP-activated (CFTR) Cl^- channels and epithelial Ca^{2+}-activated Cl^- channels were found to be ineffective in preventing apoptosis.[21] These results clearly underline the importance of Cl^- extrusion most likely through a volume-regulated chloride channel during the early phase of apoptosis upstream of the mitochondria.

In hematopoietic cells after induction of apoptosis by hyperosmotic shock, staurosporine or Fas ligation, oligonucleosomal (DNA laddering) but not high molecular weight (50-150 kbp) DNA degradation was abolished by preventing Cl^- efflux.[40] The efflux was prevented by a raise in extracellular Cl^- or by application of Cl^- channel blockers (NPPB, ddFSK). However, other apoptotic parameters such as PARP cleavage, chromatin condensation and nuclear envelope disruption were still observed. These data suggest that a reduction of intracellular Cl^- through ORCC is required for caspase-activated DNAse (CAD) activation but not for execution of programmed cell death. Additionally, in a rat liver cell line (HTC), apoptosis triggered with TNF-α elicited a 5-fold increase in Cl^- current that was sensitive to NPPB and DPC (Cl^- channel blockers). Furthermore, substitution of Cl^- in the medium also abolished the TNF-α induced Cl^- current. Interestingly, a TNF-induced K^+ current was also inhibited by Cl^- removal but not by Cl^- channel blockers, suggesting that the K^+ channel opening does not depend on Cl^- movement. However, Cl^- binding might affect K^+ channel kinetics. The channel opening was also Ca^{2+}, ATP and PKC, but not calmodulin kinase II, dependent.[33]

In conclusion, these data in concert indicate that a loss of intracellular chloride is important for the apoptotic signaling cascade either directly by enabling activation of caspases and nucleases or indirectly by affecting plasma membrane potential and/or intracellular pH. The efflux of Cl^- possibly occurs through a volume-regulated chloride channel in the early phase of apoptosis upstream of mitochondria.

6. SODIUM MOVEMENT DURING THE APOPTOTIC RESPONSE

As described above, potassium and chloride have been the primary focus in most studies examining the role and movement of intracellular ions during apoptosis. The significance of these ions during apoptosis is due mainly to the high concentration of intracellular potassium that occurs in most mammalian cells and chloride's ability to act as a counter ion during the cell death process. In contrast, the normally low concentration of intracellular sodium has not afforded this ion the same attention as those for potassium in relation to cell shrinkage or AVD. Thus, studies examining a role for sodium during apoptosis have lagged behind. Recently, however, a defining role for changes in intracellular sodium during apoptosis has been reported specifically in regard to cell shrinkage and the AVD process.

Early studies on ions and apoptosis have suggested a change in intracellular sodium as part of the programmed cell death process. Treatment of peripheral-blood human T-lymphocytes with nanomolar concentrations of *Staphylococcus aureus* alpha-toxin gave rise to small membrane pores that permitted movement of monovalent, but not divalent, ions.[41] This permeabilization and subsequent ion movement resulted in characteristics of apoptosis including internucleosomal DNA degradation. Interestingly, treatment of human T-lymphocytes with low concentrations of alpha-toxin in the absence of extracellular sodium did not permit the occurrence of internucleosomal DNA cleavage, suggesting a role for Na^+ ions in precipitating the programmed cell death process. Additionally, in a study of dopamine-induced apoptosis in mouse thymocytes, an increase in intracellular sodium using X-ray microanalysis of cellular elements was observed.[42] Furthermore, in studies involving oxidized low-density lipoprotein (LDL) treatment of human monocytes and UV-irradiated human monoblastoid cells (U937), X-ray microanalysis of total element content suggested a higher sodium content in cells undergoing apoptosis which occurred prior to the loss of membrane integrity.[43, 44] Thus, these early studies on ion movement during apoptosis have implied a critical role for sodium flux during the programmed cell death process.

Our work has shown that multiple apoptotic stimuli induce an increase in intracellular sodium that occurs prior to the loss of membrane integrity.[45] In this study, Jurkat T-cells treated with an anti-Fas antibody, the calcium ionophore A23187, or thapsigargin showed a time-dependent increase in intracellular sodium that occurred not only prior to the loss of membrane integrity but also preceded the loss of cell volume. Additionally, this sodium influx was reflected in depolarization of the plasma membrane that again occurred prior to cell shrinkage. Using flow cytometry to examine cells that had not shrunk at the single-cell level, a direct correlation between cells with increased intracellular sodium and a depolarized plasma membrane was observed.[45] In primary rat thymocytes, dexamethasone was shown to induce plasma membrane depolarization both *in vivo* and *in vitro*, in a time- and dose-dependent manner.[46] The cellular depolarization of thymocytes was shown to be a direct effect of glucocorticoid-induced apoptosis since HeLa cells, which contain a functional glucocorticoid receptor but do not die in response to hormone, did not alter their plasma membrane potential in response to steroid treatment.

Once the apoptotic stimulated cells depolarize, this change in plasma membrane potential was sustained throughout the cell death process, suggesting an inability of dying cells to repolarize to a normal membrane potential. Further study into anti-Fas treated Jurkat cells showed that the ability of these cells to maintain cellular depolarization was

due in part to an early inhibition/degradation of the Na^+/K^+-ATPase.[45, 47] Inhibition of the Na^+/K^+-ATPase would not only facilitate an increase in intracellular sodium and membrane depolarization, but also promote a loss of intracellular potassium through loss of the cells primary K^+ uptake mechanism. Furthermore, the addition of the cardiac glycoside ouabain that inhibits the Na^+/K^+-ATPase was shown to enhance anti-Fas induced apoptosis in Jurkat cells.[45, 48] Treatment of cultured cortical neurons or human prostatic smooth muscle cells with ouabain alone results in both necrotic and apoptotic components depending not only on the cell type, but also on the concentration of ouabain employed.[49, 50] Additionally, cardiac glycosides, including ouabain, were shown to induce apoptosis in androgen-independent human prostate cancer cell lines.[51]

A critical role for the Na^+/K^+-ATPase in its efforts to maintain ionic homeostasis during apoptosis has also been described in several other studies [reviewed by Yu[52]]. In early studies, the onset of apoptosis in freshly isolated proximal tubule cells was accompanied by a decline in Na^+/K^+-ATPase activity.[53] Treatment of mouse cortical neurons with low concentrations of ouabain resulted in a slight loss of Na^+/K^+-ATPase activity but did not affect potassium homeostasis or cell viability.[54] However, in combination with non-lethal doses of various apoptotic stimuli such as C_2-ceramide or -amyloid, low concentrations of ouabain induced apoptosis, suggesting that slight impairment of the Na^+/K^+-ATPase and disruption of potassium homeostasis can lead to the apoptotic cascade. In a different study involving cultured cortical neurons, Na^+/K^+-ATPase activity was directly suppressed by apoptotic insults including serum deprivation, staurosporine, and C_2-ceramide.[55] Additionally, these authors suggested preserving pump activity might provide a neuroprotective effect in certain pathological conditions. In contrast, studies in porcine renal proximal tubular cells, cultured rat cerebellar granule cells, and vascular smooth muscle cells suggest that inhibition of the Na^+/K^+-ATPase protects cells from apoptosis,[56-58] implying the role of the Na^+/K^+-ATPase during programmed cell death may be cell-type specific.

We have recently defined an important role for sodium flux in controlling AVD during apoptosis.[59] Treatment of Jurkat cells with anti-Fas in the presence of sodium-substituted media abolished the increase in intracellular sodium and resulted in cellular swelling which is characteristic of necrosis. However, further characterization of these swollen, anti-Fas treated cells revealed numerous traits associated with apoptosis including chromatin condensation, externalization of the phosphatidylserine, caspase activation and activity, along with internucleosomal DNA degradation. Interestingly, a loss of intracellular potassium was observed in the swollen cells that accompanied the apoptotic events. The addition of sodium back into the extracellular environment led to the loss of cell volume and the typical apoptotic morphology associated with apoptosis. Therefore, apoptotic cell shrinkage can be uncoupled from other programmed cell death characteristics, thus defining specific roles for both sodium and potassium; sodium controlling cell size while potassium controls the apoptotic machinery.

7. CONCLUSION AND PERSPECTIVES

The understanding of programmed cell death or apoptosis has become one of the most studied and fastest growing fields in the life science area. The implication of volume regulatory mechanisms, ion channels as well as ion movements, are recognized

as playing an important role during the activation of programmed cell death and for the regulation of the cell shrinkage characteristic of apoptosis. The knowledge gained through decades of studying cellular volume regulation and the mechanisms counterbalancing osmo-changes provide a great resource in the current application to identify and characterize apoptosis related ion fluxes and regulated ion channels. Although many similarities between RVD and AVD are known**,** obvious differences exist between these two responses. Understanding the mechanisms regulating apoptosis related volume changes and the ion movements resulting in the activation of the cell death program may lead to the development of drugs that target ion channels or transporters and tip the balance between cell life and death.

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