Uncoupling Cell Shrinkage from Apoptosis Reveals That Na\(^+\) Influx Is Required for Volume Loss during Programmed Cell Death*

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Cell shrinkage, or the loss of cell volume, is a ubiquitous characteristic of programmed cell death that is observed in all examples of apoptosis, independent of the death stimulus. This decrease in cell volume occurs in synchrony with other classical features of apoptosis. The molecular basis for cell shrinkage during apoptosis involves fluxes of intracellular ions including K\(^+\), Na\(^+\), and Cl\(^-\). Here we show for the first time that these ion fluxes, but not cell shrinkage, are necessary for apoptosis. Using sodium-substituted medium during anti-Fas treatment of Jurkat cells, we observed cellular swelling, a property normally associated with necrosis, in contrast to the typical cell shrinkage. Surprisingly, these swollen cells displayed all of the other classical features of apoptosis, including chromatin condensation, externalization of phosphatidylserine, caspase activity, poly(ADP)-ribose polymerase cleavage, and internucleosomal DNA degradation. These swollen cells had a marked decrease in intracellular potassium, and subsequent inhibition of this potassium loss completely blocked apoptosis. Reintroduction of sodium ions in cell cultures reversed this cellular swelling, resulting in a dramatic loss of cell volume and the characteristic apoptotic morphology. Additionally, inhibition of sodium influx using a sodium channel blocker saxitoxin completely prevented the onset of anti-Fas-induced apoptosis in Jurkat cells. These findings suggest that sodium influx can control not only changes in cell size but also the activation of apoptosis, whereas potassium ion loss controls the progression of the cell death process. Therefore cell shrinkage can be separated from other features of apoptosis.

Apoptosis is a mode of cell death defined by unique biochemical and morphological components that include cell shrinkage, nuclear condensation, caspase activation, poly(ADP)-ribose polymerase (PARP)\(^1\) cleavage, and DNA fragmentation (1–3). The shrinkage of cells is a universal feature of apoptosis that is completely conserved among species ranging from Caenorhabditis elegans to man, regardless of the apoptotic stimuli. When apoptotic cells shrink, a significant loss of intracellular potassium, sodium, and chloride occurs (4–13) that is necessary for maximal caspase activation and optimal apoptotic nuclelease activity (7, 8, 14–17). Maintenance of the normal physiologic intracellular concentration of these monovalent ions has been shown to inhibit apoptosis and thus can serve as a repressive mechanism during programmed cell death (7, 18–20). Recently, we showed that a variety of apoptotic stimuli result in an early transient increase in intracellular sodium associated with a marked plasma membrane depolarization that occurs prior to the loss of cell volume and other characteristics of apoptosis (23). Although this increase in intracellular sodium appears to occur in part because of an early inhibition of the Na\(^+\)/K\(^-\)\textsuperscript{-}-ATPase during anti-Fas-induced apoptosis, the precise role for this sodium influx is not completely known.

In healthy cells, a balance of intracellular potassium and sodium ions is required not only to maintain a constant cell size but also for the overall viability of the cell. Variations in the cellular ionic composition are normally compensated via inherent volume regulatory mechanisms, suggesting the importance of maintaining this balance in ionic content (24–27). Changes in the concentration of intracellular potassium and chloride have been a primary focus of studies examining apoptotic cell shrinkage. The high intracellular concentration of potassium in most mammalian cells has made this ion an attractive target in dissecting the consequence of cell shrinkage during apoptosis. However, the exact mechanism that accounts for the loss of intracellular potassium during apoptosis and subsequent cell shrinkage has not been identified, although it has been suggested that inhibition of the Na\(^+\)/K\(^-\)\textsuperscript{-}-ATPase may be one of the mechanisms contributing to the eventual potassium efflux and loss of cell volume during apoptosis (23, 28).

In contrast, studies elucidating a role for intracellular sodium during apoptosis have lagged behind its aforementioned intracellular cation counterpart, despite the critical function sodium plays in controlling and maintaining a homeostatic cell volume. To date no evidence, either genetic or biochemical, has been reported separating cell shrinkage from other characteristics of apoptosis. Here we report that ion fluxes, but not cell shrinkage, are necessary for apoptosis. We show that sodium influx is necessary for cell shrinkage but not for the activation of the cell death effectors. In contrast, potassium efflux is critical for death by apoptosis, regardless of changes in cell size. Additionally, complete inhibition of sodium influx results in a delay in the activation of the apoptotic program, suggesting that sodium may have multiple roles during programmed cell death.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Jurkat cells, E6.1 (human lymphoma) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 4 mM glutamine, 31 mg/liter penicillin, and 50 mg/liter streptomycin at 37 °C, 7% CO\(_2\) atmosphere. Induction of apoptosis in Jurkat cells (5 × 10\(^5\) cells/ml) was accomplished using 10 ng/ml anti-human Fas IgM (Kamiya Biomedical). The cells were incubated at 37 °C, 7% CO\(_2\) atmosphere for various periods of time. For choline- or NMDG-substituted

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‡ The abbreviations used are: PARP, poly(ADP)-ribose polymerase; NMDG, N-methyl-d-glucamine; PBBF-AM, potassium-binding benzofuran isophthathate acetoxymethylester; SBFI-AM, sodium-binding benzofuran isophtathate acetyloxymethylester; PI, propidium iodide; DIC, differential interference contrast; FITC, fluorescein isothiocyanate.
Intracellular Ionic Regulation of Apoptosis

Increased Intracellular Sodium Occurs prior to the Loss of Cell Volume—We initially examined Jurkat cells at the single-cell level for changes in intracellular sodium using the fluorescent SBFI-AM (Na+) dye and flow cytometry. Gating on only the normally sized or nonshrunken cells, anti-Fas treatment in the presence of SBFI-AM (Na+) resulted in two distinct populations of cells: one with a control level of intracellular sodium and one with a Na+ level for changes in intracellular sodium by flow cytometry using the fluorescent sodium SBFI-AM (Na+) The cells were initially analyzed by gating on only the normal or nonshrunken cells on a forward scatter versus side scatter dot plot. Subsequently, this population of cells was then analyzed on a forward scatter (cell size) versus SBFI (Na+) fluorescence dot plot and on an SBFI (Na+) fluorescence histogram. An increase in SBFI-AM (Na+) fluorescence indicates an increase in intracellular sodium. Only the anti-Fas-treated cells showed an increase in intracellular sodium, indicating the early occurrence of this apoptotic characteristic prior to cell shrinkage.

Western Blot Analysis—Western blot analysis for PARP cleavage was accomplished by running 50 μg/sample of protein extract on a 4–20% polyacrylamide gel (Novex/Invitrogen, Carlsbad, CA) and transferring the samples to nitrocellulose. The membrane was incubated with primary anti-PARP antibody (Transduction Labs, Lexington, KY) overnight at a concentration of 500 ng/ml and then subsequently incubated with an anti-mouse secondary antibody (Amersham Biosciences) following the manufacturer’s recommended protocol. Visualization was achieved on film with the ECL chemiluminescent system (Amersham Biosciences).

DNA Analysis by Flow Cytometry—The DNA content for each sample was determined by flow cytometry by pelleting 3–5 ml of cells from both control and anti-Fas-treated samples. The cells were fixed by the slow addition of cold 70% ethanol to a volume of ~1.5 ml, adjusted to 5 ml with cold 70% ethanol, and stored at 4 °C overnight. For flow analysis, the fixed cells were pelleted, washed once in 1× phosphate-buffered saline, and stained in 1 ml of 20 μg/ml of PI, 1 mg/ml RNase in 1× phosphate-buffered saline for 20 min. 7500 cells were analyzed by flow cytometry using a Becton Dickinson FACSort by gating on a PI area versus width dot plot to exclude cell debris and cell aggregates. The percentage of degraded DNA was determined by the number of cells with subdiploid DNA divided by the total number of cells examined under each experimental condition.

DNA Analysis by Agarose Gel—DNA was isolated by pelleting 2.0–5.0 × 106 cells followed by resuspension in 500 μl of 5 mM Tris (pH 7.4), 20 mM EDTA, and 0.5% Triton X-100. The samples were stored at ~20 °C overnight. The cell lysates were thawed and treated with 0.4 mg/ml proteinase K overnight at 55 °C. The samples were then extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). NaCl was added to a final concentration of 0.1 M, and the DNA was precipitated by the addition of 2 volumes of ice-cold ethanol overnight at ~80 °C. The DNA was pelleted, dried, and resuspended in 100 μl of 10 mM Tris, 1 mM EDTA buffer to which 0.1 mg/ml deoxyribonuclease-free ribonuclease (RNase) was added. The samples were incubated overnight at 37 °C. DNA concentrations were determined by absorbance at 260 nm, and 15 μg of DNA were examined on a 1.8% agarose gel stained with ethidium bromide.

Sodium Replenishment Assay—Jurkat cells cultured in sodium-substituted choline medium were treated with and without 50 ng/ml of an anti-Fas antibody for 4 h at 37 °C, 7% CO2 atmosphere. After this time, the cells were harvested and resuspended back into either fresh sodium substituted choline medium or normal RPMI 1640 medium, the latter of which contained the standard levels of extracellular sodium. The cells were incubated for an additional 30 min at 37 °C, 7% CO2 atmosphere. Morphological examination of these cells was performed using DIC microscopy.

RESULTS

Analysis of Caspase Activity by Flow Cytometry—Caspase activity was determined using the CaspTag activity kit (Intergen, Purchase, NY) for the fluorescent detection of caspase-3, -8, and -9 according to the manufacturer’s instructions. Briefly, 1.5 × 106 control or treated cells were added to the various caspase substrates and incubated at 37 °C, 7% CO2 for 30 min. Immediately prior to flow cytometric examination, PI was added to each sample at a final concentration of 10 μg/ml. Ten thousand cells were analyzed using a BD FACSsort flow cytometer with an excitation of 488 nm and emission at 530 and 585 nm for caspase activity and PI, respectively.

Analysis of DNA Ploidy by Flow Cytometry—DNA content for each sample was determined by flow cytometry using a Becton Dickinson FACSort by gating on a PI area versus width dot plot to exclude cell debris and cell aggregates. The percentage of degraded DNA was determined by the number of cells with subdiploid DNA divided by the total number of cells examined under each experimental condition.

Analysis of Cellular Membrane Lipid Symmetry by Flow Cytometry—Intracellular Sodium and Potassium Measurements, 2 μl of 2.5 mM SBFI-AM (Na+) or PBFI-AM (K+) (Molecular Probes) stock were individually added to 1 ml of cells at a final concentration of 5 μM 1 h prior to the time of examination. Incubation was continued at 37 °C, 7% CO2 atmosphere. Immediately prior to flow cytometric examination, propidium iodide (PI) (Sigma) was added to a final concentration of 10 μg/ml. Ten thousand cells were analyzed by sequential excitation of the cells containing SBFI-AM and PI at 340–350 and 488 nm, respectively, using a FACSVantage SE flow cytometer (Becton Dickinson) and CellQuest software.

Microscopy—A laser scanning confocal microscope (LSM 410 NLO—Axioplan, Carl Zeiss, Inc.) was used to observe differential interference contrast (DIC) images. The images were obtained simultaneously using the 488-nm line from the included argon ion laser as the light source and the Zeiss Plan-Apo 100 ×/1.4 objective lens. The software used for acquisition was Zeiss LSM 510 NT, version 3.2. Immediately following acquisition, the images were analyzed with LSM Image Examiner (licensed) version 2.0 for Windows NT. Hoechst stained cells were examined on a LSM 510 UV mounted on an Axiovert 200M microscope. (Carl Zeiss, Inc.) with a Plan-Apo 63 ×/1.4 objective lens to obtain simultaneous DIC and UV images. Hoechst was excited at 361 nm from an Enterprise 653 laser (Coherent Laser, Auburn, CA), and emission was collected with a 395LP filter. The pinhole was adjusted so that the slice thickness in Z was ~6 μm.

The DNA content for each sample was determined by flow cytometry by pelleting 3–5 ml of cells from both control and anti-Fas-treated samples. The cells were fixed by the slow addition of cold 70% ethanol to a volume of ~1.5 ml, adjusted to 5 ml with cold 70% ethanol, and stored at 4 °C overnight. For flow analysis, the fixed cells were pelleted, washed once in 1× phosphate-buffered saline, and stained in 1 ml of 20 μg/ml of PI, 1 mg/ml RNase in 1× phosphate-buffered saline for 20 min. 7500 cells were analyzed by flow cytometry using a Becton Dickinson FACSort by gating on a PI area versus width dot plot to exclude cell debris and cell aggregates. The percentage of degraded DNA was determined by the number of cells with subdiploid DNA divided by the total number of cells examined under each experimental condition.
and one with increased intracellular sodium (Fig. 1). Previous kinetic studies revealed that the cell population with increased intracellular sodium correlated with those that had depolarized their plasma membrane, and it is these cells that proceed to adopt the characteristic features of apoptosis (23). Interestingly, these cells remained depolarized, even after cell shrinkage, in part because of an inhibition of the Na\(^+\)/H\(^+\)/K\(^+\)/H\(^+\)-ATPase, thus preventing their cellular repolarization (23). However, this inhibition of the Na\(^+\)/K\(^+\)-ATPase alone does not account for the elevated level of sodium seen in the nonshrunken, anti-Fas-treated cells (data not shown), suggesting that additional ion transport processes exist to account for the increase in intracellular sodium. These transport mechanisms may be intimately linked to the activation of the programmed cell death process.

**Prevention of Na\(^+\) Influx Induced Cell Swelling**—To examine the consequence of this early increase in intracellular sodium during apoptosis, we exposed Jurkat cells to sodium-substituted medium, thus removing the normal concentration gradient or driving force for sodium influx, while maintaining isotonic conditions. Standard RPMI 1640 medium that contains 125 mM Na\(^+\) was substituted with sodium-free components. Because complete removal of sodium from this medium by dialyzing the fetal calf serum was incompatible with life, the cells were cultured in a small amount of sodium that was present in normal heat-inactivated fetal calf serum. Flow cytometry analysis of intracellular sodium using SBFI-AM (Na\(^+\)) versus PI fluorescence dot plot. The data are shown as SBFI-AM (Na\(^+\)) histograms. The histograms are representative of a single experiment, and the graph represents averages of five independent experiments. B, changes in cell size was initially determined by measuring the cells ability to scatter light in the forward direction by flow cytometry. An increase or decrease in forward light scatter indicates an increase or decrease in cell size, respectively. The white lines on the plots denote the peak light scatter values under each individual control condition and were used as references for determining changes in forward light scatter. The histograms represent one of at least three independent experiments. C, morphological examination of control and anti-Fas treated Jurkat cells cultured in normal or sodium-substituted medium using DIC microscopy. Classical apoptotic morphology was observed for cells treated with anti-Fas under normal conditions. A swollen cellular morphology was observed in anti-Fas-treated Jurkat cells in either sodium-substituted choline or NMDG medium. D, anti-Fas-treated Jurkat cells under normal and sodium-substituted conditions examined for chromatin condensation with Hoechst 33342. Simultaneous DIC and Hoechst detection showed the occurrence of swollen cells in the sodium-substituted choline or NMDG medium that had condensed chromatin, similar to anti-Fas-treated Jurkat cells under normal sodium conditions.
Numerous apoptotic characteristics occur in sodium-substituted choline or NMDG medium during anti-Fas induced apoptosis. A, externalization of phosphatidylserine was determined using FITC-conjugated annexin V by flow cytometry. Flow cytometric analysis of externalization of phosphatidylserine showed an increase in the number of cells that had an increase in annexin V-FITC fluorescence prior to the loss of membrane integrity (lower right-hand quadrant). The dot plots are representative of a single experiment, and the graph represents the averages of five independent experiments. B, caspase activity for caspase-3, -8, and -9 was determined using CaspaseTag activity kits and flow cytometry. The cells were initially examined on a forward scatter versus PI dot plot to analyze only the viable cells. An increase in fluorescence indicates an increase in caspase activity. The graph represents the averages of three independent experiments. C, Western blot analysis for PARP cleavage. Protein extracts from Jurkat cells cultured in either normal or sodium-substituted medium were examined using an antibody to PARP. The presence of the 24-kDa protein band indicates cleavage of the PARP protein. D, examination of DNA content by flow cytometry. The occurrence of degraded DNA is shown below the G0 DNA peak (white line). The histograms represent a single experiment, and the numbers on the plots are the average number of cells with subdiploid DNA from five independent experiments. E, DNA isolated from control and anti-Fas-treated Jurkat cells under normal, sodium-substituted choline, or NMDG conditions were examined by agarose gel electrophoresis. Under each condition the occurrence of internucleosomal DNA cleavage was observed characteristic of apoptosis.
presence of sodium-substituted choline or sodium-substituted NMDG medium showed an unexpected change in cell size by flow cytometry. Under normal sodium conditions, an increase in the number of cells that had a diminished capacity to scatter light in the forward direction, indicative of cells with a smaller or shrunken cell size typical of apoptosis (29), was observed in Jurkat cells treated with anti-Fas (Fig. 2B). However, upon treatment of cells with anti-Fas in sodium-substituted medium, an increase in forward scatter light was observed, indicative of cellular swelling (Fig. 2B). Interestingly, control cells cultured in either sodium-substituted medium had a slightly decreased capacity to scatter light in the forward direction compared with the control cells cultured in normal medium, suggesting that the entire population of cells was adjusting their resting cell volume. Morphological examination of Jurkat cells treated with anti-Fas for 6 h in normal RPMI 1640 medium on a confocal microscope with Nomarski optics showed shrunken cells with cellular blebbing, characteristic of apoptosis (Fig. 2C). In marked contrast, Jurkat cells treated with anti-Fas in either sodium-substituted choline or sodium-substituted NMDG medium exhibited a swollen morphology, classically associated with necrosis (Fig. 2C). However, these swollen cells had condensed chromatin and apoptotic nuclei, indicative of the apoptotic morphology as observed by Hoechst staining (Fig. 2D).

**Apoptosis Can Occur without Cell Shrinkage**—Based on these findings, we sought to determine whether other apoptotic characteristics occurred in the swollen anti-Fas-treated Jurkat cells cultured in sodium-substituted medium. Externalization of phosphatidylserine on the cell surface was examined using annexin V-FITC and flow cytometry. Treatment of Jurkat cells under normal conditions with anti-Fas resulted in flipping of phosphatidylserine prior to the loss of membrane integrity, characteristic of apoptosis (Fig. 3A). Similarly, Jurkat cells cultured in either sodium-substituted choline or sodium-substituted NMDG medium also exhibited phosphatidylserine externalization to an equivalent extent as observed under normal medium conditions (Fig. 3A), suggesting that this feature of apoptosis can occur in the absence of extracellular sodium and cell shrinkage. Additionally, anti-Fas-treated cells cultured in the presence of normal or sodium-substituted medium were evaluated for activation of various caspases associated with apoptosis (30–32) using fluorescent substrates for caspase-3, -8, and caspase-9-like enzymes and flow cytometry in living cells. Under normal medium conditions, treatment of Jurkat cells with anti-Fas resulted in an increase in caspase-3, -8, and caspase-9-like activity (Fig. 3B). When cells treated with anti-Fas were cultured in either sodium-substituted choline or sodium-substituted NMDG, comparable activation of all three caspases was observed (Fig. 3B), suggesting that sodium influx and cell shrinkage are not required for caspase activation and/or activity. To access the downstream functionality of caspase activity, we examined these cells for the cleavage of PARP, a constitutive nuclear protein involved in DNA repair and a well characterized substrate for caspase-3-like proteases. Western blot analysis of control protein extracts from normal or sodium-substituted medium showed no occurrence of PARP cleavage (Fig. 3C). However, in anti-Fas-treated extracts, degradation of the PARP protein was observed under all conditions (Fig. 3C). These data indicate that not only are caspases active in the swollen “apoptotic” cells, but they are both functional and targeted to their appropriate subcellular location.

It has been well documented that internucleosomal DNA fragmentation, characteristic of apoptosis, is completely restricted to the shrunken apoptotic cells (7, 8, 33). Therefore, we examined anti-Fas-treated cells cultured under normal or sodium-substituted conditions for changes in their DNA integrity by flow cytometry. Jurkat cells treated with anti-Fas under normal extracellular sodium conditions exhibited the presence of a subdiploid peak of DNA, indicating the occurrence of degraded DNA (Fig. 3D). Similar results were observed in cells cultured in either sodium-substituted choline or sodium-substituted NMDG medium (Fig. 3D). The nature of this DNA fragmentation was explored by agarose gel electrophoresis. The presence of internucleosomal DNA cleavage, a hallmark characteristic of apoptosis, was evident in Jurkat cells treated with anti-Fas in either normal or sodium-substituted medium (Fig. 3E). Our analysis of programmed cell death in the absence of the normal concentration of sodium indicates that although this ion is critical for cell shrinkage during apoptosis, sodium is not necessary for the generation of other apoptotic traits. Thus, we show for the first time that cell shrinkage can be uncoupled from the programmed cell death process.

**Loss of Intracellular Potassium Controls the Activation of the Apoptotic Machinery**—The loss of intracellular potassium has been shown to play a key role in the activation of the apoptosis, and elimination of the normal electrochemical gradient of this ion has been shown to block apoptosis. To determine whether changes in intracellular potassium occurred in anti-Fas-treated cells cultured under sodium-substituted conditions, we used the fluorescent PBFI-AM (K⁺) dye and flow cytometry. Treatment of Jurkat cells with anti-Fas under normal sodium conditions resulted in a marked decrease in intracellular potassium (Fig. 4). Interestingly, when anti-Fas-treated Jurkat cells were cultured in either sodium-substituted choline or NMDG medium, a similar decrease in intracellular potassium was also observed (Fig. 4). This finding suggests that the loss of intracellular potassium is not solely dependent upon an increase in intracellular sodium, indicating that independent
High extracellular potassium blocks the apoptotic machinery in sodium-substituted medium during anti-Fas-induced apoptosis. Jurkat cells under control sodium-substituted or sodium-substituted high K⁺ conditions were examined for changes in intracellular K⁺ (A), externalization of phosphatidylserine (B), caspase-3 activity (C), and DNA degradation by flow cytometry (D). The presence of high external K⁺ attenuated all of these apoptotic characteristics. High external K⁺ alone had no effect on any of these apoptotic traits in the absence of anti-Fas. The histograms and contour plots represent one of at least three independent experiments.
Intracellular Ionic Regulation of Apoptosis

The presence of extracellular sodium controls apoptotic volume decrease. Jurkat cells initially cultured in sodium-substituted choline medium and placed back into fresh sodium-substituted medium retained their swollen morphology (top panels). Jurkat cells initially cultured in sodium-substituted choline medium and placed back into normal medium that contained the standard concentration of sodium showed a shrunken morphology characteristic of apoptosis (bottom panels).

Fig. 6. The presence of extracellular sodium controls apoptotic volume decrease. Jurkat cells initially cultured in sodium-substituted choline medium and placed back into fresh sodium-substituted medium retained their swollen morphology (top panels). Jurkat cells initially cultured in sodium-substituted choline medium and placed back into normal medium that contained the standard concentration of sodium showed a shrunken morphology characteristic of apoptosis (bottom panels).

Ionic pathways can and do exist for the movement of sodium and potassium ions during programmed cell death.

Based on the above data, we speculated that this loss of intracellular potassium, even in the swollen cells, might account for the engagement of the apoptotic machinery. To examine this hypothesis, we treated Jurkat cells with anti-Fas in sodium-substituted choline or NMDG medium where the normal concentration of choline or NMDG components and KCl were switched while maintaining isotonicity. These conditions establish a high potassium (102.7 mM), low sodium-substituted environment. Jurkat cells cultured in either sodium-substituted choline or NMDG high K+ medium were indistinguishable from cells cultured in the normal sodium-substituted medium in regards to their intracellular potassium (Fig. 5A). Treatment of Jurkat cells with anti-Fas under sodium-substituted high K+ conditions resulted in the inhibition of intracellular potassium loss (Fig. 5A), similar to observations made in normal sodium, high K+ medium (7, 8, 19, 21). Markedly, the presence of high extracellular K+ attenuated the generation of other apoptotic characteristics, including externalization of membrane phosphatidylserine, caspase-3 activity, and DNA degradation even in the sodium depleted medium (Fig. 5, B–D). Thus, the loss of intracellular potassium plays a pivotal role in the engagement of apoptosis, regardless of the presence or absence of extracellular sodium.

Replacement of Extracellular Sodium Induces Cell Shrinkage and Characteristic Apoptotic Morphology—To define the precise role sodium has in orchestrating cell shrinkage during apoptosis, sodium was replenished in Jurkat cells treated with anti-Fas in sodium-substituted medium after a period of time followed by analysis for changes in cell size. As shown earlier, an increase in cell size or a swollen morphology was observed in Jurkat cells treated with anti-Fas initially under sodium-substituted conditions, which remained upon further culture in sodium-substituted medium (Fig. 6, top panels). However, after reincubation of these cells in medium that contained the normal levels of extracellular sodium, the anti-Fas-treated cells exhibited a shrunken morphology characteristic of apoptosis (Fig. 6, bottom panels). These data elucidate a crucial role for sodium influx in activating the apoptotic volume decrease response or cell shrinkage during apoptosis. Our data also show that changes in cell size are reversible upon reintroduction of sodium, whereas other features of the cell death process are regulated by the loss of intracellular potassium.

Saxtoxin Blocks Anti-Fas-induced Apoptosis—Finally, we wanted to determine the effect of sodium channel inhibition on preventing the early increase in intracellular sodium during apoptosis using the sodium channel blocker saxtoxin. The addition of saxtoxin alone had no significant effect on Jurkat cells, because these cells were similar to control (Fig. 7, compare first column with second column). When Jurkat cells were treated with anti-Fas for 6 h, we observed an increase in intracellular sodium, the loss of intracellular potassium, along with a shrunken cell population by flow cytometry (Fig. 7, third column). Additionally, these cells had degraded their DNA, all characteristic of apoptosis (Fig. 7, third column). Interestingly, the addition of the sodium channel blocker saxtoxin completely prevented the increase in intracellular sodium during anti-Fas treatment (Fig. 7, fourth column). As a result of this inhibition of sodium influx, anti-Fas-treated cells did not undergo apoptosis within 6 h, as evidenced by their failure to lose intracellular potassium, shrink, or degrade their DNA (Fig. 7, fourth column). This suggests that sodium influx may also play a role in the activation of the apoptotic program. Together, these data indicate that both early (apoptotic activation) and downstream apoptotic processes (cell shrinkage) can be prevented in the absence of sodium influx and further suggests that ionic fluxes can control or regulate programmed cell death.

DISCUSSION

Our data reveal novel insight into the effects specific ions have on the apoptotic process. Specifically our results show for the first time that cell shrinkage can be separated from other characteristics of apoptosis, with sodium controlling cell size and potassium regulating the progression of the apoptotic process. The importance of the early increase in intracellular sodium was revealed by studies where sodium-substituted medium was used to remove the concentration gradient of this ion. Under this condition, the cells induced to undergo apoptosis swell, rather than shrink, suggesting that the influx of extracellular sodium plays a key role in controlling cell size. Unexpectedly, these swollen cells still exhibited classical apoptotic traits including externalization of phosphatidylserine, caspase activation, PARP cleavage, internucleosomal DNA cleavage, and the loss of intracellular potassium. Inhibition of potassium loss using high external potassium medium completely prevented apoptosis, suggesting potassium as the key intracellular ion that holds the apoptotic death machinery in check. Thus, although cellular swelling has been a recognized hallmark of necrosis, we show that the characteristics associated with apoptosis remain intact in response to anti-Fas treatment in sodium-substituted conditions despite cellular swelling. Furthermore, replenishment of sodium into the sodium-substituted condition restores the ability of anti-Fas-treated cells to shrink, showing that sodium controls cell shrinkage during apoptosis. Interestingly, if the rise in intracellular sodium is prevented using the sodium channel blocker saxtoxin, a complete inhibition of apoptosis is observed. Thus, the rise in intracellular sodium not only appears to play a role in controlling changes in cell size but also appears to have an effect in the initial signaling of the apoptotic program.

A specific change in cell size has traditionally been a defining characteristic to discriminate apoptosis from necrosis, with the direction of the change being the key; apoptotic cells shrink, and necrotic cells swell. In fact the term “shrinkage necrosis” was originally used to describe a distinctly different mode of cellular death with features of an active, controlled process (34, 35). This process termed apoptosis was shown to play a role in cell turnover in many adult tissues, as well as during normal embryonic development, and to occur spontaneously in untreated malignant neoplasms (1). Thus, for the deletion of unwanted cells
with little or no tissue disruption, a shrunken cell is well suited for a role in maintaining cellular homeostasis. There have always been debates in terms of the importance of specific events associated with apoptosis. For example, whether mitochondrial membrane depolarization or the release of cytochrome c is a necessary feature for inducing apoptosis, whether apoptosis is controlled by caspases or is caspase-independent, and earlier as to whether internucleosomal DNA fragmentation was a decisive event required for the activation of the cell death process. However, in all well characterized examples of apoptosis, the cells always shrink. Our ability now, for the first time, to uncouple this event from other characteristics of apoptosis demonstrates that it is not the morphological observation that is important but the underlying movement of ions that holds the key to understanding how this mode of cell death is triggered.

The increase in intracellular sodium reported here that occurs prior to the loss of cell volume during apoptosis is transient, and as the cells shrink a loss of both sodium and potassium is observed. Several studies, along with ours, have suggested an early increase in intracellular sodium during apoptosis (4, 23, 36–38). Our ability here to prevent the onset of apoptosis using the sodium channel blocker saxitoxin shows that programmed cell death can be inhibited at the level of the plasma membrane. Additionally, this result suggests that an orchestrated movement of ions plays an important role in the overall programmed death process. Interestingly, in our studies using sodium congeners such as choline or NMDG show that cell shrinkage is not necessary for the progression of apoptosis. A primary difference between the saxitoxin and sodium-substituted studies is the presence of a small amount of sodium in the sodium-substituted medium from the fetal calf serum. In media where dialyzed heat-inactivated fetal calf serum was used, we found this to be incompatible with life, because control cells incubated in the complete absence of extracellular sodium loss membrane integrity and demonstrated cellular swelling (data not shown). Thus, our data suggest that the small amount of sodium contributed by the fetal calf serum, although not present at a significant level to induce cell shrinkage upon apoptotic stimulation, is sufficient to allow downstream catabolic events. Additionally, our studies show that the route of sodium entry across the plasma membrane is not destroyed during the cell death process, because reintroduction of extracellular sodium permits the cells to shrink and display traditional apoptotic morphology.

The mechanisms responsible for the cellular swelling in sodium-substituted medium and consequent potassium loss under sodium-substituted conditions are not completely understood, although under normal conditions both inhibition of potassium uptake and activation of potassium efflux appear to be involved (23, 28). Introduction of a known inhibitor for the regulatory volume decrease response, i.e. cytochalasin B, did not prevent the loss of intracellular potassium during Fas-induced apoptosis in sodium-substituted medium (data not shown), suggesting the

Fig. 7. Early increase in intracellular sodium and the onset of apoptosis is prevented with saxitoxin. Jurkat cells treated in the presence and absence of anti-Fas with and without the sodium channel blocker saxitoxin (STX) were analyzed for changes in intracellular sodium (SBFI-AM (Na+)), intracellular potassium (PBFI-AM) (K+), cell size, and DNA content (PI) by flow cytometry. A decrease in PBFI-AM (K+) fluorescence indicates a decrease in intracellular potassium. The occurrence of degraded DNA characteristic of apoptosis is shown below the G1 DNA peak (white line). The changes in intracellular ions and degraded DNA were prevented in the anti-Fas-treated cells in the presence of saxitoxin. The histograms are representative of two to three independent experiments.
absence of an active ionic efflux mechanism. This result is not surprising, because it has been suggested that multiple pathways may exist for the loss of intracellular potassium during cell death depending on the cell type and/or apoptotic stimulus (39). In this regard, it is interesting to note that UV-treated Jurkat cells in sodium-substituted medium also have a swollen morphology with the loss of intracellular potassium, suggesting that this response is not specific to Fas-induced apoptosis and more importantly that a common mechanism for the loss of this ion may exist for the loss of intracellular potassium during cell death depending on the cell type and/or apoptotic stimulus (39). In this concern, it is interesting to note that UV-treated Jurkat cells in sodium-substituted medium also have a swollen morphology with the loss of intracellular potassium, suggesting that this response is not specific to Fas-induced apoptosis and more importantly that a common mechanism for the loss of this ion may exist for the loss of intracellular potassium during cell death depending on the cell type and/or apoptotic stimulus (39).

In conclusion, this study uncouples for the first time the morphological occurrence of cell shrinkage from other apoptotic traits, revealing distinct roles for sodium and potassium ion fluxes during apoptosis. Additionally, our work shows that cell volume changes during apoptosis are reversible. Although sodium-free conditions do not exist under these conditions, it is interesting to note that UV-treated Jurkat cells in sodium-substituted medium also have a swollen morphology with the loss of intracellular potassium, suggesting that this response is not specific to Fas-induced apoptosis and more importantly that a common mechanism for the loss of this ion may exist for the loss of intracellular potassium during cell death depending on the cell type and/or apoptotic stimulus (39). In this concern, it is interesting to note that UV-treated Jurkat cells in sodium-substituted medium also have a swollen morphology with the loss of intracellular potassium, suggesting that this response is not specific to Fas-induced apoptosis and more importantly that a common mechanism for the loss of this ion may exist for the loss of intracellular potassium during cell death depending on the cell type and/or apoptotic stimulus (39).

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