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A Primary Role for K^+ and Na^+ Efflux in the Activation of Apoptosis*

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Cell shrinkage is a major characteristic of apoptosis, but the mechanism and role of this process in cell death are poorly understood. The primary factor that controls volume regulation in all cells is ions, and thus we have examined the movement of ions at the single cell level in lymphocytes during apoptosis. Activation of the death program with several stimuli that act through independent pathways to stimulate apoptosis results in a synchronous shift of cells from a normal cell size to a shrunken cell size. Only the shrunken cells exhibit DNA fragmentation and an approximate 4-fold elevation of caspase-3-like activity. Analysis of K^+ and Na^+ ion content of individual cells by flow cytometry revealed that the intracellular ionic strength of apoptotic cells decreased substantially from their non-shrunken counterparts. Additionally, we show apoptosis is enhanced under conditions where the intracellular K^+ concentration is diminished and that apoptosis is inhibited when K^+ efflux is prevented. These data show that the efflux of ions, primarily potassium, plays a necessary and perhaps a pivotal role in the cell death program.

Apoptosis is a physiological mode of cell death that removes unwanted cell populations at a specific time or in response to a given signal (1). A major characteristic of the apoptotic process is the shrinkage of cells (2, 3). This distinctive feature of volume loss in apoptotic cells is in direct contrast to the cellular swelling that occurs during necrosis. Cell shrinkage has been reported in all well characterized cases of apoptosis, suggesting a fundamental role for cell volume loss during this form of cell death. However, the mechanism of cell volume loss and its precise role in the activation of apoptosis has not been studied. We and others (4–7) have previously shown that glucocorticoid treatment of mouse, rat, and human lymphocytes results in the generation of two distinct populations of cells, one of normal cell size and one of a reduced cell size. DNA fragmentation characteristic of apoptosis occurred only in glucocorticoid-treated cells that exhibited a shrunken morphology (7). Modulation of cell volume has been shown to regulate cellular metabolism and gene expression, and changes in cell volume have been suggested to act as a second or even a third messenger during various cellular processes (8). Thus, changes in cell volume and ion content could be critical components of apoptosis.

Cell volume is directly related to the movement of ions, with homeostasis being achieved by a balance of osmotic pressure across the plasma membrane. When the concentration of solute particles on each side of the membrane is equal, a net movement of water is inhibited, thus maintaining a constant cell size. Most cells achieve and maintain this osmotic balance through the continuous activity of the Na^+/K^+ ATPase pump, which creates and maintains an intracellular environment high in potassium and low in sodium (9, 10). In contrast, the extracellular environment typically contains low levels of potassium and high levels of sodium. Despite the negative transmembrane potential, a net electrochemical gradient is established that favors the passive movement of potassium out of the cell. Therefore, the movement of ions, specifically potassium, is a likely candidate responsible for the loss of cell volume during apoptosis.

Several recent studies have implicated a role for potassium in apoptosis. For example, cytokine withdrawal from cultured human eosinophils resulted in cell shrinkage that could be partially blocked by the potent K^+ channel blocker 4-aminopyridine (11). A decrease in intracellular potassium concentration was observed during both glucocorticoid treatment of human leukemic cells and etoposide treatment of fibroblasts (12, 13). However, in all these studies, the entire population of cells was analyzed while only a small percentage of cells was undergoing apoptosis, thus resulting in a potential underestimation of ion content changes in the subpopulation of apoptotic cells. We have now used flow cytometry to analyze the relationship between cell shrinkage, DNA fragmentation, and caspase activity when apoptosis is triggered by a variety of stimuli that act via different signal transduction pathways. Additionally, we have examined intracellular K^+ and Na^+ during apoptosis at the single cell level by combining fluorescent ion indicators with flow cytometry. Our data shows that an efflux of ions, specifically K^+, is necessary for the progression of apoptosis.

MATERIALS AND METHODS

Cell Culture—S49 Neo cells (S49.1 mouse lymphoma cells) and Jurkat cells (human lymphoma) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 4 mM glutamine, 75 units/ml streptomycin, and 100 units/ml penicillin at 37 °C, 7% CO_2 atmosphere. S49 Neo cells were treated with 0.25 μM dexamethasone or 0.4 μM anisomycin for 24 h, 2 μM A23187 or 0.5 μM thapsigargin for 8 h, or 0.05 μM staurosporin for 6 h to induce apoptosis. Jurkat cells were treated with 10 ng/ml anti-human Fas IgM (Kamiya Biomedical) for 5 h. For hypotonic medium, RPMI 1640 media was diluted (1:1) in distilled water and supplemented as described above. For high potassium medium, RPMI 1640 media was initially made minus KCl and NaCl. The KCl concentration was adjusted to 102.7 mM (the normal NaCl concentration of RPMI 1640) and the NaCl concentration was adjusted to 5.4 mM (the normal KCl concentration of RPMI 1640) and supplemented as described above.

Flow Cytometry—Flow cytometric analysis to determine DNA content was accomplished as described previously (7). Seven thousand five hundred cells were examined by flow cytometry for each sample using

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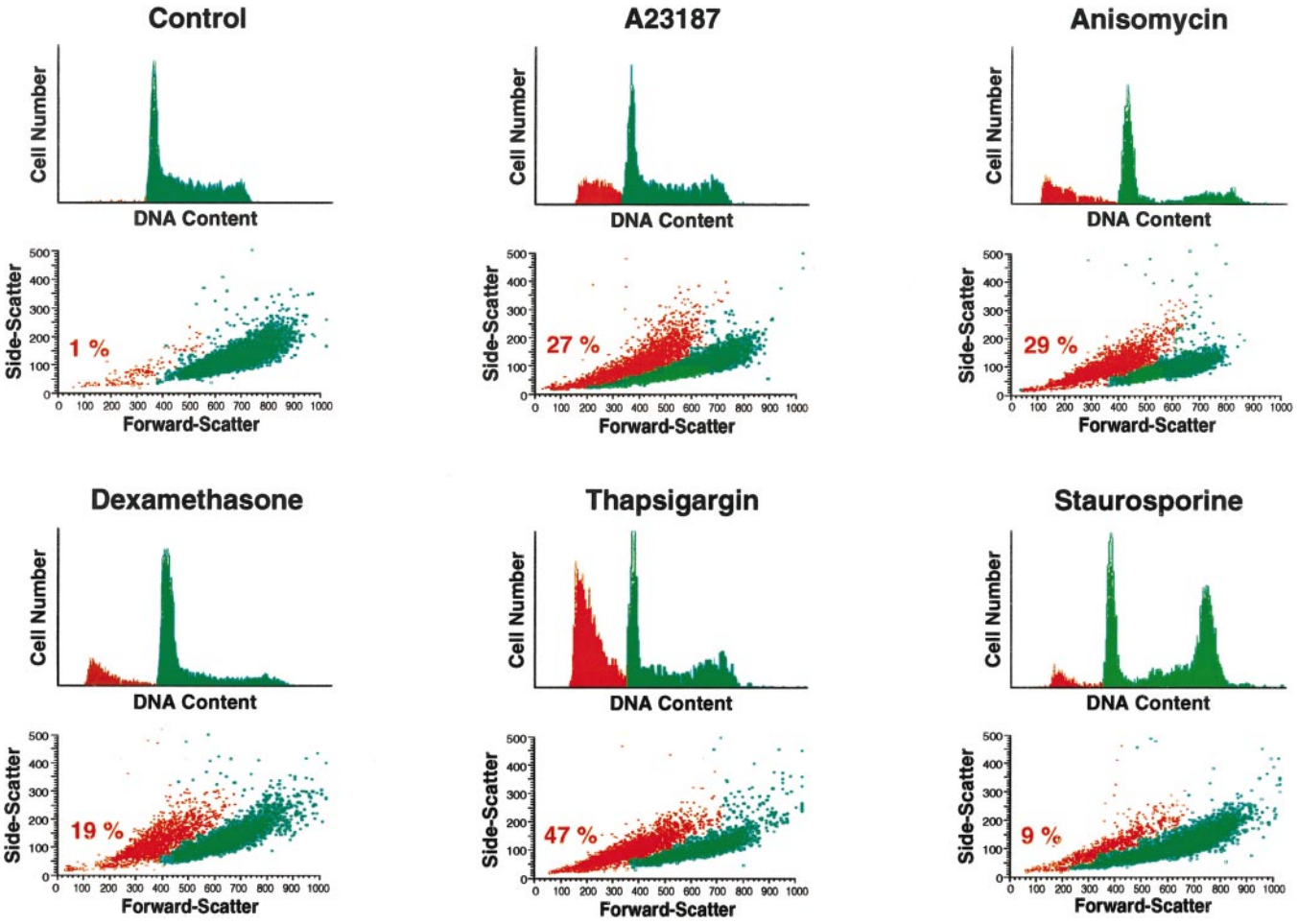

FIG. 1. **Relationship between cell volume and DNA degradation.** S49 Neo cells were treated with various apoptotic agents, fixed, and then examined by flow cytometry. Analysis of the DNA content and light scattering properties of 7500 control cells showed essentially a normal DNA content profile and a single population of cells on a forward scatter *versus* side scatter dot plot. Gates set on a PI-based area *versus* width dot plot were used to separate and analyze the DNA content in this sample to determine the position of the cells with various DNA integrity on the light scatter dot plot by multi-color analysis. An initial gate was set on the single population of control cells observed on the area *versus* width dot plot. A second gate was set for cells that have a reduced area and width. These gates were then also used to examine cells treated with the various apoptotic agents. Analysis of the DNA content in the control sample showed that cells with a normal DNA profile had a large cell size (shown in *green*), and the small proportion of cells in the subdiploid region of the DNA content histogram, representing degraded DNA, had a small or shrunken cell size (shown in *red*). The number in *red* shows the percent of shrunken cells with degraded DNA. Upon treatment with various apoptotic stimuli, the number of cells comprising the subdiploid region of the DNA content histogram increased and directly correlated to the position of the smaller size cells on the forward scatter *versus* side scatter dot plots. Therefore, only the shrunken population of cells contained DNA which had been degraded. Data shown are representative of three identical experiments.

a Becton Dickinson FACSort, and the cells were excited using a 488-nm argon laser. To examine the K^+ and Na^+ content of viable apoptotic cells, S49 Neo and Jurkat cells treated in the presence or absence of various apoptotic agents were loaded with either the potassium- or sodium-sensitive fluorescent dyes potassium-binding benzofuran isophthalate (PBFI-AM)[1] and sodium-binding benzofuran isophthalate (SBFI-AM) (Molecular Probes), respectively. One hour prior to the time of harvest, each group of cells was split into 2 equal volumes, and half was loaded with either PBFI-AM or SBFI-AM to a final concentration of 5 μM. Stock solutions of both dyes (2.5 mM) were prepared fresh by combining equal volumes of a 25% (w/v) Pluronic F-127 (Molecular Probes) and Me_2SO. After 1 h of loading the fluorescent dyes, the cells were harvested and resuspended in 1 ml of RPMI 1640 and then stored on ice. Flow cytometry was accomplished using a Becton Dickinson FACStar. Ten thousand cells were analyzed by excitation of the dyes at 340–350 nm, and the emission was examined at 425 nm. In studies incorporating propidium iodide (PI), the cells after the final harvest were resuspended in 1 ml of RPMI 1640 containing 10 μg/ml PI prior to putting on ice.

Cell Sorting—Simultaneous sorting of both the high PBFI and low PI fluorescence population (normal) from the low PBFI and low PI fluorescence population (apoptotic) of cells was accomplished using a Becton Dickinson FACStar. Gates were set on either a PBFI (K^+) *versus* PI fluorescence dot plot or a forward scatter *versus* side scatter dot plot. Cells were sorted into 3 ml of RPMI 1640 medium and placed on ice.

Cell Volume—The average volume per cell of various cell populations was determined by electronic sizing using a Coulter Multisizer II, Coulter Electronics Ltd. Cells between 5 and 20 μm in size were analyzed. The total cell volume of a population of cells was divided by the total number of cells to determine the average volume per cell.

Calculations for Determining Intracellular Potassium Concentration—An initial intracellular potassium concentration of 140 mM and an initial cell volume of 100% was used to calculate the intracellular potassium concentration in the shrunken or apoptotic cell. Our study has shown a 90% decrease in the potassium content using PBFI-AM coupled to a 40% loss of cell volume in the shrunken, apoptotic population of cells. To determine the final intracellular potassium concentration in the shrunken population of apoptotic cells, the initial potassium concentration was multiplied by the initial cell volume which was then divided by the final cell volume times the percent of potassium that remained in the cell. Calculations based the cell diameter, the loss of potassium, and the movement of water molecules per potassium ion efflux yielded identical results.

Measurement of Protease Activity—Caspase-3-like activity was measured using a fluorometric assay (14).

[1] The abbreviations used are: PBFI-AM, potassium-binding benzofuran isophthalate-acetoxymethyl ester; SBFI-AM, sodium-binding benzofuran isophthalate-acetoxymethyl ester; PI, propidium iodide.

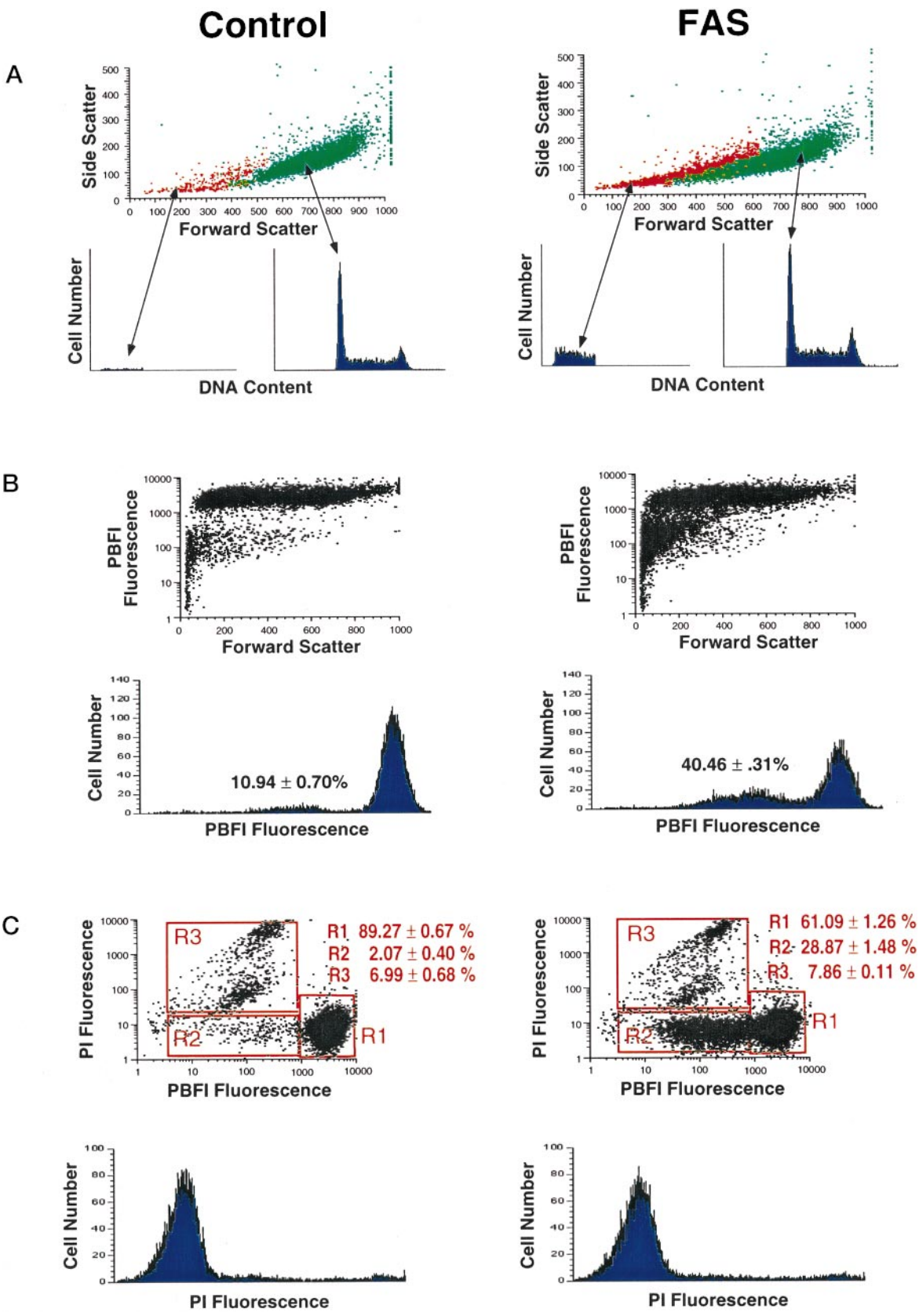

FIG. 2. **Jurkat cells treated with the anti-Fas antibody.** Jurkat cells were treated with and without 10 ng/ml anti-Fas antibody for 5 h. *A*, cells fixed in 70% ethanol and stained with PI were examined by flow cytometry. Analysis of the DNA content and light scattering properties for control cells showed essentially a normal DNA content with a single population of cells on a forward scatter *versus* side scatter dot plot. Cells treated with the anti-Fas antibody showed an increase in the number of cells containing subdiploid DNA and two populations of cells on the light scatter plot: one of normal (control) cell size (shown in *green*), and one of a reduced or shrunken cell size (shown in *red*), containing degraded DNA similar to what was observed for the apoptotic S49 Neo cells (see Fig. 1). *B*, analysis of the PBFI-AM (K^+) fluorescence in control and anti-Fas-treated Jurkat cells showed a major decrease in PBFI-AM fluorescence or potassium content in the shrunken population for the anti-Fas-treated cells. Histograms showing the number of cells at a given PBFI-AM fluorescence showed a 30% increase in the percent of cells that

TABLE I

PBFI-AM (K^+, top) and SBFI-AM (Na^+, bottom) flow cytometric analysis of Jurkat cells treated with 10 ng/ml anti-Fas antibody for 5 h

The percent of normal size or shrunken cells ± S.E. was determine from region 1 (R1) and region 2 (R2), respectively, over four independent experiments. PBFI-AM (K^+) or SBFI-AM (Na^+) fluorescence was the mean fluorescence ± S.E. averaged from four independent experiments.

Agent	% Cells		PBFI-AM (K^+)	
	Normal Size	Shrunken	Normal Size	Shrunken
Control	89.3 ± 0.6	2.1 ± 0.4	1504 ± 139	124 ± 22
Anti-Fas	61.1 ± 1.1	28.8 ± 1.3	1209 ± 116	98 ± 20
Agent	% Cells		SBFI-AM (Na^+)	
	Normal Size	Shrunken	Normal Size	Shrunken
Control	91.2 ± 1.6	2.4 ± 0.6	43 ± 15	5 ± 1
Anti-Fas	64.1 ± 4.1	30.4 ± 3.9	62 ± 4.3	4 ± 0.1

TABLE II

Caspase-3-like activity in individually sorted normal and shrunken populations of Jurkat cells after 5 h of anti-Fas treatment

Results represent the mean caspase activity ± S.E. from three independent experiments.

	Caspase-3-like activity
	pmol AFC/min/mg
Normal	67.3 ± 6.8
Shrunken	240.4 ± 22

TABLE III

PBFI-AM (K^+, top) and SBFI-AM (Na^+, bottom) flow cytometric analysis of S49 Neo cells treated with either 0.25 μM dexamethasone or 0.4 μM anisomycin for 24 h; 2 μM A23187 or 0.5 μM thapsigargin for 8 h; or 0.05 μM staurosporin for 6 h

Ten thousand cells were analyzed under each condition. The percent of normal size or shrunken cells ± S.E. were determine from region 1 (R1) and region 2 (R2), respectively, on the PBFI-AM (K^+) or SBFI-AM (Na^+) *versus* PI fluorescence dot plots averaged from three independent experiments. PBFI-AM (K^+) or SBFI-AM (Na^+) fluorescence is the mean fluorescence ± S.E. averaged from three independent experiments.

Agent	% Cells		PBFI-AM (K^+)	
	Normal Size	Shrunken	Normal Size	Shrunken
Control	92.6 ± 0.6	3.7 ± 0.9	1951 ± 180	27 ± 2
Dex	58.8 ± 2.4	16.6 ± 2.2	1740 ± 130	31 ± 3
Aniso	17.4 ± 1.5	39.1 ± 3.9	1630 ± 118	22 ± 1
A23187	33.0 ± 10	23.2 ± 4.4	1885 ± 235	36 ± 7
Thaps	56.4 ± 2.8	11.0 ± 2.5	1731 ± 182	40 ± 7
Stauro	82.2 ± 5.8	5.0 ± 1.5	1507 ± 311	27 ± 4
Agent	% Cells		SBFI-AM (Na^+)	
	Normal Size	Shrunken	Normal Size	Shrunken
Control	91.2 ± 1.3	3.7 ± 1.0	176 ± 24	24 ± 1
Dex	56.0 ± 3.2	15.8 ± 4.0	176 ± 27	24 ± 2
Aniso	17.9 ± 1.7	34.2 ± 4.0	147 ± 13	17 ± 1
A23187	30.9 ± 7.1	22.0 ± 6.2	148 ± 8.9	28 ± 1
Thaps	54.3 ± 3.6	7.8 ± 2.0	147 ± 22	27 ± 1
Stauro	80.7 ± 5.9	5.0 ± 2.3	161 ± 16	27 ± 2

RESULTS AND DISCUSSION

Examination of control S49 Neo lymphoma cells by flow cytometry on a forward scatter *versus* side scatter dot plot revealed essentially a single population of cells that had a normal DNA content as judged by PI staining (Fig. 1). When apoptosis was activated by a variety of agents, a subdiploid peak of DNA characteristic of apoptosis was observed (Fig. 1). Similarly, two distinct populations of cells were observed on dot plots of forward *versus* side scatter light. Analysis of cells that had a normal DNA content (shown in *green*) on a plot of forward scatter *versus* side scatter light revealed a population of cells similar to the untreated controls (Fig. 1). Analysis of cells that had degraded their DNA to apoptotic subdiploid levels (shown in *red*) revealed a population of cells with a decreased forward scatter and increased side scatter of light, indicative of shrunken cells. Comparison of the data from several apoptotic stimuli known to act via distinct signal transduction pathways showed that only the shrunken population of cells under each apoptotic condition has degraded their DNA. Thus, a positive relationship exists between the loss of cell volume and DNA degradation induced through a variety of apoptotic pathways.

We extended this study to include Jurkat cells, widely recognized to undergo apoptosis upon engagement of the Fas receptor (15–17), to further examine the relationship between cell size and DNA content by flow cytometry during apoptosis. Treatment of these cells with an antibody against the Fas receptor also resulted in the generation of two distinct populations: one of normal DNA content and cell size (shown in *green*), and one with degraded DNA and reduced cell size (shown in *red*; Fig. 2*A*). Therefore, the relationship between cell size and DNA content also exists for this receptor-activated cell death pathway.

We next used the anti-Fas-treated Jurkat cells as a model system to study the movement of ions during apoptosis. Flow cytometric analysis of these cells loaded with the cell-permeant acetoxymethyl ester derivative of the fluorescent potassium-sensitive dye PBFI-AM (18) also revealed two distinct populations of cells on a forward scatter (cell size) *versus* PBFI-AM fluorescence (potassium content) dot plot (Fig. 2*B*). The major population contained cells of a larger size with a high degree of PBFI-AM (K^+) fluorescence, representing the normal, non-apoptotic cells. A second population of cells was also observed with a shrunken cell size and lower PBFI-AM (K^+) fluorescence. The amount of PBFI-AM (K^+) fluorescence in the normal cell population was relatively constant over the range of cell sizes observed in the forward scatter *versus* PBFI-AM (K^+) plot, indicating K^+ fluorescence was independent of normal size variations. Anti-Fas treatment of Jurkat cells resulted in approximately 30% more cells exhibiting a 10-fold decrease of PBFI-AM (K^+) fluorescence (Fig. 2*B*). Co-analysis of these two independent parameters showed that the population of cells with decreased PBFI-AM (K^+) fluorescence corresponded precisely to the shrunken population of cells which contained degraded DNA, characteristic of apoptosis.

In this model system, and other models of apoptosis, cell death is highly asynchronous. A late occurrence during programmed cell death is the loss of membrane integrity (19), an event which compromises the ability to accurately measure intracellular ions. To determine whether the loss in PBFI-AM (K^+) fluorescence observed in the apoptotic cells had occurred prior to a loss in membrane integrity, cells were treated with PBFI-AM and PI, since PI can only enter dead cells (20). Analysis of these cells by two-color flow cytometry revealed three distinct populations of cells (Fig. 2*C*). One population (R1) had

have potassium loss, averaged over four independent experiments. The presence of two populations of cells observed on the forward scatter *versus* PBFI-AM (K^+) fluorescence dot plots are similar to the separation observed on a forward scatter *versus* side scatter dot plot. *C*, analysis of PBFI-AM (K^+) fluorescence *versus* propidium iodide (*PI*) dot plots showed a striking increase in the percent of cells that have potassium loss and have low PI fluorescence (intact plasma membrane) in the anti-Fas-treated Jurkat cells averaged over four independent experiments. PI fluorescence histograms showed no increase in the number of cells that have lost their membrane integrity.

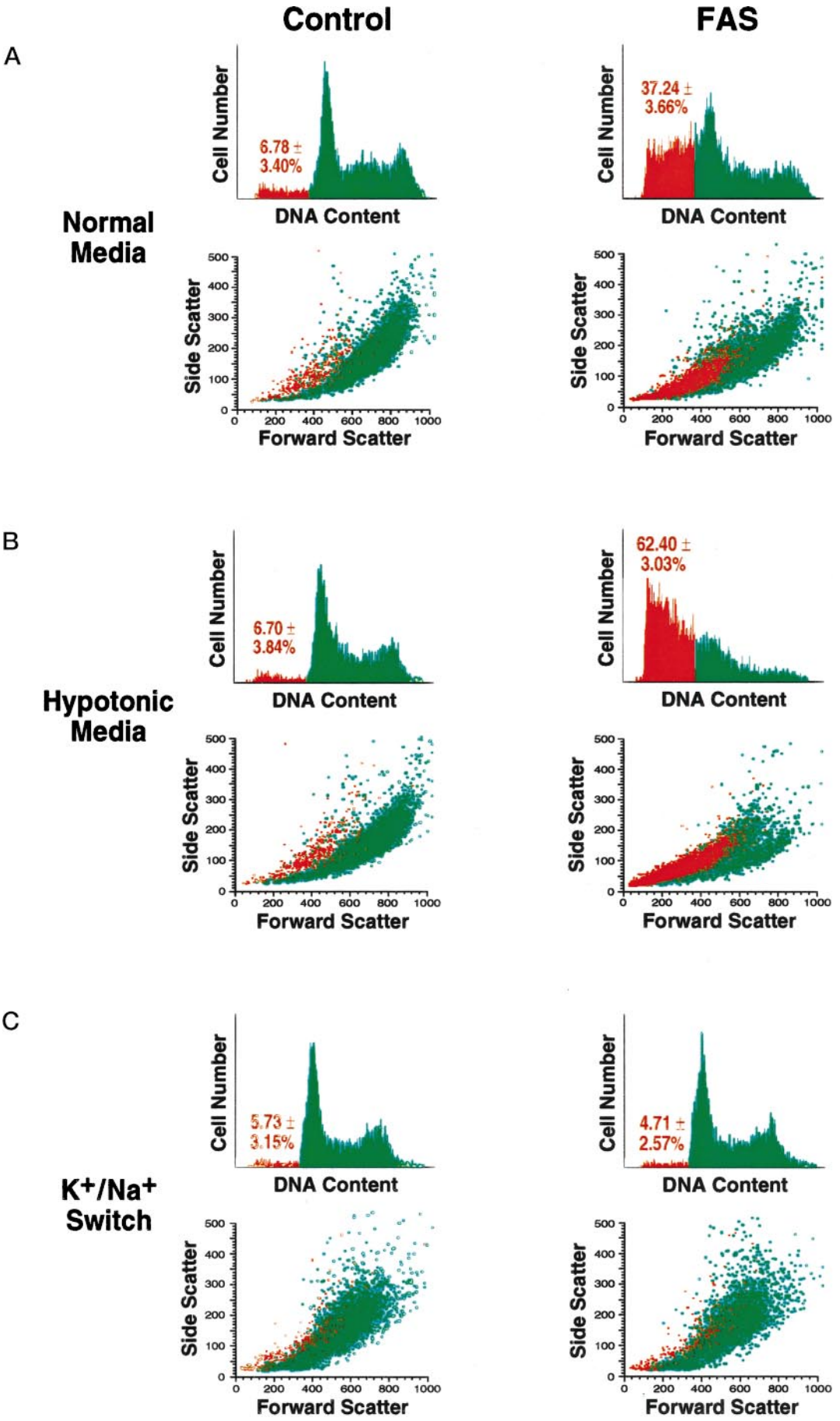

FIG. 3. **Jurkat cells treated with and without the anti-Fas antibody under normal, hypotonic, or increased extracellular potassium conditions.** Plots shown are representative of a single study, and the percentages are the average of four independent experiments. *A*,

a high PBFI-AM (K^+) and low PI fluorescence corresponding to the normal viable population of cells. A second population (R2) had a decreased PBFI-AM (K^+) and low PI fluorescence, corresponding to early apoptotic cells. Finally, a third population (R3) exhibited a decreased PBFI-AM (K^+) and elevated PI fluorescence, corresponding to dead cells that were excluded from further ion analysis. Clearly, however, the majority of cells with a reduction in PBFI-AM (K^+) fluorescence have an intact cell membrane.

Quantitation of PBFI-AM (K^+) fluorescence in Jurkat cells undergoing apoptosis is shown in Table I. Anti-Fas treatment of Jurkat cells resulted in a 30% increase in the number of shrunken cells that had an intact cell membrane as judged by PI staining (Fig. 2*C*) and trypan blue exclusion (data not shown). Quantitation of data obtained from several experiments showed that these shrunken cells had a 92% reduction in PBFI-AM (K^+) fluorescence when compared with the normal or non-shrunken population of cells. Individually sorting and electronically sizing the population of cells with a normal PBFI-AM (K^+) and a low PI fluorescence (R1) from cells with a reduced PBFI-AM (K^+) and low PI fluorescence (R2) revealed that the shrunken population of cells had a 40% decrease in cell volume. Thus, the 90% decrease in potassium content in cells having a 40% reduction in volume would yield an intracellular potassium concentration of less than 50 mM. This concentration of potassium measured with the fluorescent dye is consistent with values obtained in other systems (12, 13) and in our own studies using inductively coupled plasma/mass spectrometry (14). The data in Table I also show that the normal size population of anti-Fas-treated cells exhibited a small decrease in PBFI-AM (K^+) fluorescence compared with untreated control cells, indicating that the loss of potassium likely causes the reduction in cell volume during apoptosis. Interestingly, a small population of untreated Jurkat cells also exhibited a marked reduction in potassium concentration comparable to that observed in anti-Fas-treated cells. Microscopic examination of these untreated Jurkat cells revealed that these represent a small population of naturally apoptotic cells. Thus, if uncompensated by other ions, the intracellular ionic strength of Jurkat cells undergoing apoptosis is significantly lower than nondying cells.

Because of the large change in PBFI-AM (K^+) fluorescence observed in the shrunken population of anti-Fas-treated Jurkat cells, we wondered if the apoptotic cells compensated with an influx of Na^+ which can also regulate cell volume. Individual cell sodium content was measured with the cell-permeant acetoxymethyl ester derivative of the fluorescent sodium-sensitive dye SBFI-AM (18) and PI. Three populations of cells were also observed in these studies, which were similar to those seen with the potassium indicator. The total fluorescence detected with the sodium indicator was invariably lower than that of the potassium indicator. Cells that had lost their membrane integrity were excluded from analysis. Table I shows that a major 89% loss in the mean SBFI-AM (Na^+) fluorescence occurs in the anti-Fas-treated Jurkat cells. Because intracellular sodium exists at approximately 12 mM, or one-twelfth the level of intracellular potassium, the loss of this ion is less likely to contribute toward both the reduction in cell volume and in decreasing the overall intracellular ionic strength of the apoptotic cell. These data reveal a major reduction in both K^+ and Na^+ concentrations only in the shrunken population of Jurkat cells that have fragmented their DNA, characteristic of apoptosis. To determine if early events in the apoptotic cascade, such as caspase activity (21, 22), were restricted to the shrunken population of cells, we sorted the shrunken, apoptotic population of anti-Fas-treated Jurkat cells from the normal, non-shrunken population, revealing a nearly 4-fold increase in caspase-3-like activity in the shrunken population (Table II). This relationship between cell shrinkage, ion loss, and caspase activity is also observed when caspase activity is analyzed in live cells. Only cells that shrink exhibit caspase activity *in vivo*. Preliminary studies with the caspase inhibitor zVAD-fmk revealed that this inhibitor can block anti-Fas-induced shrinkage, although not shrinkage induced by other apoptotic signals.[2] Thus, several critical components of apoptosis occur only in cells that have shrunken and lost ionic strength.

To determine if similar changes in K^+ and Na^+ occur in other cells induced to undergo apoptosis, we examined the intracellular ions in murine S49 Neo cells which also undergo apoptosis in response to a variety of agents (see Fig. 1). Induction of apoptosis in S49 Neo cells through these diverse apoptotic pathways revealed three populations of cells when examined on a PBFI-AM (K^+) *versus* PI fluorescence dot plot (data not shown). Cells that had lost membrane integrity were again excluded from analysis. The data in Table III, along with Fig. 1, indicate that cells exhibiting shrunken apoptotic morphology and DNA degradation have a major reduction in intracellular K^+ and Na^+ content. Thus the loss of K^+ and Na^+ during apoptosis occurs across animal species and is independent of the mode of activation of apoptosis.

A critical question generated by our observations is whether K^+ efflux is necessary for the activation and progression of apoptosis. To address this issue, we exploited the volume regulatory mechanisms in lymphocytes to modulate the intracellular K^+ concentration and K^+ efflux during apoptosis. When Jurkat cells were exposed to the anti-Fas antibody for 5 h under isotonic conditions, approximately 37% of the cells degraded their DNA and were shrunken (Fig. 3*A*). Culture of lymphocytes under hypotonic conditions immediately expands cell size due to an increase in intracellular water. These cells then compensated for this increase in cell volume by the activation of a regulatory volume decrease response and lose potassium and chloride ions, allowing for the movement of water from the cells to subsequently regain a near normal cell size (23). This volume recovery response leads to an approximately 50% reduction in potassium concentration (data not shown) but clearly does not activate apoptosis (Fig. 3*B, left panel*). Thus K^+ depletion alone, generated by exposure of cells to hypotonic conditions in the absence of an apoptotic stimulus, does not trigger apoptosis nor alter the long term viability of the cells (7). However, treatment of these hypotonically treated Jurkat cells with the anti-Fas antibody for 5 h almost doubled the number of cells undergoing apoptosis (Fig. 3*B, right panel*). Similar data were also obtained when hypotonically treated S49 Neo cells were exposed to a variety of apoptotic stimuli

[2] C. D. Bortner and J. A. Cidlowski, unpublished results.

Jurkat cells treated with the anti-Fas antibody for 5 h showed an increase in the percent of cells (37%) which degraded their DNA compared with control cells (7%) along with the concomitant increase in the number of cells with a smaller or shrunken cell size. *B*, under hypotonic conditions, where the cells initially swell then regulate their volume by the loss of potassium and chloride, the percent of anti-Fas-treated Jurkat cells that have degraded DNA in 5 h was increased by 85% compared with similar treated cells under normal (isotonic) conditions. This increase in DNA fragmentation was attributed to the hypotonic cells having a lower potassium content during the anti-Fas treatment. *C*, when the normal concentrations of sodium and potassium are switched in the external environment, limiting the amount of potassium that can be lost during apoptosis, the loss of cell volume and DNA degradation induced by anti-Fas treatment are not observed.

(data not shown). Therefore, the induction of apoptosis in cells with a reduced intracellular K^+ concentration significantly enhances the activation of the cell death program.

If the loss of intracellular potassium is necessary for a cell to undergo apoptosis, then inhibiting K^+ efflux should inhibit the programmed cell death process. To test this hypothesis, Jurkat cells treated with the anti-Fas antibody were cultured under conditions where the normal sodium and potassium concentrations in the media were switched, such that extracellular potassium was high and sodium was low. Therefore, the natural potassium electrochemical gradient is reduced, thus impeding the movement of potassium from the dying cells while maintaining extracellular osmolality. Culturing Jurkat cells in high extracellular potassium did not alter the DNA content or cell viability, although the entire population of cells was slightly smaller in size when compared with control cells. However, diminishing the normal potassium electrochemical gradient completely abrogated the ability of the anti-Fas antibody to induce apoptosis in Jurkat cells (Fig. 3*C*). Additionally, both caspase activity and the loss of the mitochondrial membrane potential are inhibited under this condition.[2] Together these data indicate that controlling the intracellular potassium level in cells can directly regulate the progression of the apoptotic process. An important implication of our observations is the fact that apoptotic cells actually become hypotonic when compared with their non-dying counterparts. Several lines of evidence suggest that this change may be a key step in the decision of cells to live or die. For example, the nucleases that degrade DNA during apoptosis as well as the activity of caspase-1 (ICE) are effectively inhibited at a physiological K^+ concentration (14, 24, 25). Similarly, *in vitro* activation of the procaspase by dATP and cytochrome *c* is effectively inhibited by physiological K^+ concentrations (14). Thus the transition of an apoptotic cell from a state of high ionic strength to low ionic strength permits both the loss in cell volume and the activation of enzymes that mediate apoptosis.

The potassium electrochemical gradient observed across the plasma membrane indicates that this ion may play a role in controlling intracellular events, similar to how the natural calcium concentration gradient leads to various intracellular signaling pathways (26). Although the gradient of potassium is not as great as for calcium, the movement of potassium still may have profound effects during apoptosis and may in turn assist in the orchestration of the programmed cell death process. This orchestration may be accomplished by the concentration of positive apoptotic factors, the physical disruption of the cell structure as the cell shrinks, the activation of enzymes that in turn could degrade inhibitory apoptotic proteins, or perhaps by facilitating protein-protein interactions that would not normally occur at higher ionic strength found in non-dying cells.

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