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Cell-Surface Exposure of Phosphatidylserine Correlates With the Stage of Fludarabine-Induced Apoptosis in Chronic Lymphocytic Leukemia and Expression of Apoptosis-Regulating Genes

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Background: Programmed cell death (PCD) is characterized by a sequence of tightly regulated events that result in the activation of caspases and in internucleosomal DNA cleavage. Late apoptotic events such as DNA-strand breaks can be assayed by in situ end labeling (ISEL) and DNA measurement (sub G1) using flow cytometry. Phosphatidylserine (PS) redistribution from the inner plasma membrane leaflet to the outer leaflet, an early event in PCD, can be detected by annexin V (AxV) binding to PS. AxV-fluorescein isothiocyanate (FITC) fluorescence intensity is variable and characterizes different cell populations, denoted here as AxV-negative (AxV^{neg}), AxV-low-positive (AxV^{lo}), and AxV-high-positive (AxV^{hi}).

Methods: We investigate the correlation of three methods (ISEL, sub G1 DNA content, and AxV assay) for detecting apoptosis with focus on differences between populations with different levels of PS. We also examined the expression of PCD-regulating Bcl-2 family members in these cell populations by reverse transcription-polymerase chain reaction (RT-PCR). Chronic lymphocytic leukemia (CLL) cells exposed to fludarabine (FAMP) were used as an in vitro model. Cells with different PS/AxV levels were separated using fluorescence-activated cell sorting (FACS).

Results: Only purified AxV^{hi} cells had high positivity in the ISEL and sub G1 assays (94 ± 0.6%, 88.6 ± 6.6%, and 98.6 ± 0.6%, respectively), indicating that late apoptotic cells are detected equally by all three methods. In the AxV^{lo} population, ISEL was positive in 21% ± 13% and DNA sub G1 in 20% ± 6.6% of cells, suggesting that AxV identifies early apoptotic cells better than the other assays. Anti-apoptotic Bcl-2 and Bcl-X_L were upregulated by FAMP when cells entered apoptosis (AxV^{lo}), as was pro-apoptotic Bcl-X_S, which was undetectable in nonapoptotic AxV^{neg} cells. Pro-apoptotic Bax was only expressed in AxV^{neg} and AxV^{lo} cells. Late apoptotic AxV^{hi} cells did not express Bcl-X_S or Bax.

Results: (1) AxV staining is more sensitive than sub G1 or ISEL in detecting early apoptotic cells; (2) only late apoptotic cells are equally detected by all assays; (3) AxV is a valuable tool in the detection and isolation of apoptotic cells at different stages of PCD; and (4) pro-apoptotic Bcl-X_S and Bax are expressed at early, not late, stages of apoptosis. Cytometry 40:19–25, 2000.

Key terms: apoptosis; annexin V; TUNEL; ISEL; CLL; fludarabine; Bcl-2; Bcl-X_S

Apoptosis, or programmed cell death (PCD), is a major physiological form of homeostasis in many cell systems that are tightly regulated (1–3). Events in apoptosis include cell dehydration, cell membrane changes, condensation of nuclear chromatin, disintegration of the nuclear envelope, mobilization of intracellular ionized calcium, a decrease of the mitochondrial transmembrane potential, activation of caspases, and internucleosomal DNA cleavage. Therefore, a wide spectrum of methods has been developed to assess and quantify apoptosis in biological systems. Late apoptotic events such as DNA-strand breaks can be assessed by the terminal deoxynucleotidyl transferase (TdT) assay. TdT uses in situ end labeling (ISEL) with biotinylated deoxyuridine triphosphate (bio-dUTP; 2,4,5) and detection of nuclear DNA fragments by flow cytometry, resulting in the appearance of cells with sub

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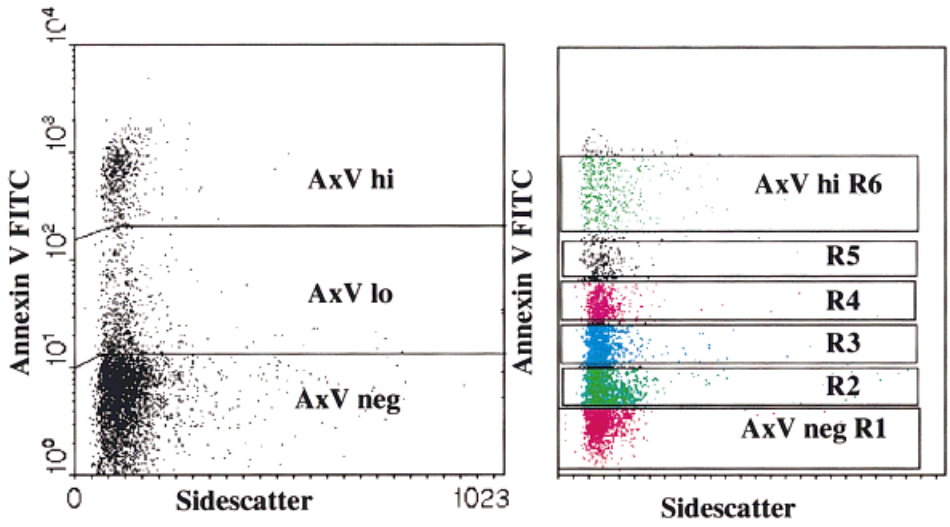

FIG. 1. **(A)** Three populations with increasing intensity of PS exposure measured by AxV binding. The marker between AxV^{neg} and AxV^{10} is set according to the negative control. **(B)** Sorting regions for AxV^{neg} , AxV^{10} (R2 through R5), and AxV^{hi}.

G1 DNA content (sub G1; 1,4,5). Redistribution of the phospholipid phosphatidylserine (PS) from the inner plasma membrane leaflet to the outer leaflet is an early apoptotic event and can be demonstrated by utilizing the binding of PS to fluorescein isothiocyanate (FITC)-conjugated annexin V (AxV) on the cell surface (6–8). The PS/AxV method allows for multiparametric analysis of live cells by flow cytometry. It has been proposed that PS expression in epithelial tumor cells is associated with early stages of apoptosis (9) and that cells with low levels of PS were apoptotic by transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay.

In hematopoietic cells, PS is involved in a variety of processes, including cell-to-cell interactions, cell activation, hemostasis, membrane fusion events, and apoptosis (10). We, therefore, studied changes in PS expression in leukemic cells exposed to the cytostatic purine analogue fludarabine (FAMP; 11). Our aim was to dissect the early stages of drug-induced apoptosis that were expected to be TUNEL negative and to correlate PS expression and DNA fragmentation with cell survival. We sorted PS-negative cells and cells expressing different levels of PS by fluorescence-activated cell sorting (FACS) and followed their survival. We observed that cells expressing low levels of PS were negative for TUNEL, exhibited a new "speckled" pattern of TUNEL positivity at intermediate PS levels, and became entirely positive for TUNEL at high PS levels. We suggest that PS/AxV is able to dissect distinct stages of apoptosis in hematopoietic cells. We demonstrated the differential expression of pro- and anti-apoptotic Bcl-2 family members in these populations.

MATERIALS AND METHODS

Cell Culture Conditions and Induction of Apoptosis

Mononuclear peripheral blood (PB) cells were obtained from patients with chronic lymphocytic leukemia (CLL) following informed consent according to institutional guidelines. Samples with white blood cell counts greater than 20,000/μl were separated by Ficoll-Hypaque density-gradient centrifugation (Sigma, St. Louis, MO) and cultured at 37°C in a humidified incubator for 24 h in RPMI supplemented with 10% heat-inactivated fetal calf serum and 5% penicillin and streptomycin (all from GIBCO BRL, Gaithersburg, MD). To induce apoptosis, FAMP, the active form of fludarabine (F-Ara-A; kindly provided by Dr. William Plunkett, The University of Texas M.D. Anderson Cancer Center), was added at a concentration of 3 μM to 4×10^6 cells/ml. Cells were evaluated at 0, 3, 5, 7, 10, 20, and 24 h to determine the expression of PS at these time points. In subsequent experiments, the 24-h time point was chosen to compare results obtained with different methods (n = 5).

PS/AxV Staining by Flow Cytometry

Cells suspended at a concentration of 4×10^6 were incubated with AxV conjugated to FITC (Apoptest-FITC; Nexins Research, Hoeven, The Netherlands) at a dilution of 1:100 in RPMI or a calcium-containing binding buffer that is provided with AxV (6). After 10 min of incubation, data were acquired using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) at 488 nm of laser light excitation and emission acquired in Fl1. Data were analyzed using the Lysis II software application (Becton Dickinson). Propidium iodide (PI) was added at a final concentration of 5 μg/ml to eliminate necrotic cells and to document the loss of membrane integrity as a phenomenon related to apoptosis.

Sorting of AxV-Stained Cells

Cells were stained as described above. Three sorting regions of increasing AxV intensity (AxV^{neg}, AxV^{lo}, and AxV^{hi}) (Fig. 1A) were set for all experiments. In two experiments, six different regions were distinguished, subdividing AxV^{lo} into four additional sorting regions with increasing staining intensity (R2 through R5, Fig. 1B). Cells were sorted into RPMI with a physiological calcium

concentration at 4°C on a FacsVantage flow cytometer. Aliquots from each region were reanalyzed after restaining with AxV-FITC, acridine orange, or ISEL. To test whether AxV staining changed during sorting, which took 3 h, an aliquot of AxV^{lo} was restained with AxV and measured immediately after acquiring 5×10^5 cells. These cells were compared with cells from the same population stained after the completion of sorting. Sorting for the polymerase chain reaction (PCR) was done accordingly.

DNA/RNA Measurements With Acridine Orange

For DNA analysis, a DNA-RNA differential staining method using acridine orange was performed as described previously (12,13). In short, 50 μl of a 1×10^6-ml cell suspension was mixed with 100 μl of a permeabilization solution and incubated for 2 min. Two hundred microliters of acridine orange solution (12 μg/ml) was added before data acquisition with a FACScan flow cytometer at 488 nm of laser light excitation and analysis with the Lysis II application. Cells were gated for cell width versus cell area to exclude doublets then gated again to exclude necrotic cell fragments.

Detection of DNA-Strand Breaks by TdT Assay

Fixation of cells was performed with BD Lyse (Becton Dickinson, Santa Cruz, CA), which contains 1.5% paraformaldehyde, for 10 min, followed by one wash in phosphate-buffered saline (PBS). Cytospins were prepared and stored at -20°C for at least 1 day for analysis. The ApopTag Plus In Situ Apoptosis Detection Kit was used (Oncor, Gaithersburg, MD). Slides were thawed to room temperature (RT), washed in PBS, resuspended in equilibration buffer for 5 min, and incubated with deoxyterminaltransferase (dTT) and digoxigenated dUTP for 1 h at 37°C in a humidified chamber. After 100 min, slides were washed three times in PBS followed by incubation with antidigoxigenin fluorescein and three washes in PBS. Slides were then counterstained with 4′6-diamidino-2-phenylindole · 2 HCl (DAPI; 0.1 μg/ml) and mounted in Vectashield Antifade mounting solution (Vector Laboratories, Burlingame, CA). Fluorescent signals were detected utilizing a Nikon microscope with a triple bandpass filter and photographs were taken with a Nikon FX-35 WA camera (Chromatechnology, Brattleboro,VT) with a magnification of 60×. All enumerations were done in a blind manner, with positive and negative controls included in each experiment.

RNA Isolation and Reverse Transcription (RT)

RNA was isolated according to the single-step acid guanidinium thiocyanate-phenol-chloroform method (14). Purified RNA samples were quantitated using a DU-640 spectrophotometer (Beckman Instruments, Fullerton, CA). An RT kit was used to synthesize cDNA, according to the manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN).

0.5 μg of the total RNA template was used per 5 μl of reverse transcriptase reaction. The primers for Bcl-2, Bcl-X_L, Bcl-X_S, Bax, and beta-2-microglobulin (B2-M) as control were synthesized on an oligonucleotide synthesizer (Applied Biosystems, Foster City, CA, model 392): B2-M A: ACCCCCACTGAAAAAGATGA, B: ATCTTCAAACCTCCATGATG; Bcl-2, A: ATGGCGCACGCTGGGAGAAC, B:CTTGTGGCTCACATAGGCAC; Bcl-X, A: AGTTTGAACTGCGGTACCGGC, B: GAACCAGCGGTTGAAGCGTTCCT; Bax, A: ATGGTCGGGTCCGGGGAGCAG, B: TCAGCCCATCTTCTTCCAGATG. Amplification and detection were performed using ^{32}P-dCTP incorporation as described (13). The radioactive products were detected and quantitated on the Betascope-603 (Betagen, Waltham, MA), after exposing the gel to Kodak film.

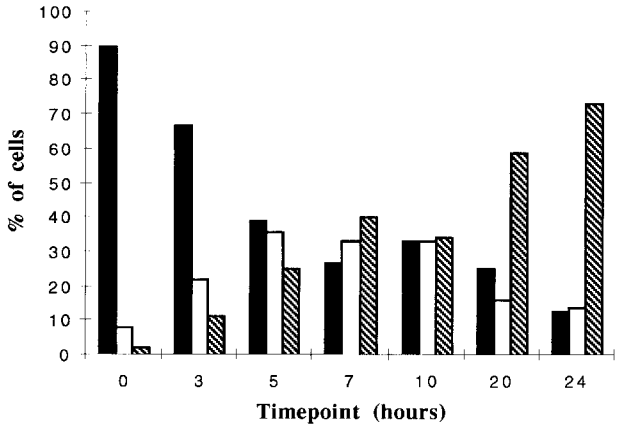

FIG. 2. Dynamics of CLL cells undergoing apoptosis when cultured with FAMP over time. Percentage of positve cells is seen on the y axis. Early apoptotic cells (AxV^{lo}) reach a steady state (time points 5, 7, and 10) over time and, as the reservoir of viable cells (AxV^{neg}) is diminished, are decreased in parallel with an increase in late apoptotic cells (AxV^{hi}). This suggests that AxV^{lo} really are cells of transition. Black columns, AxV^{neg}; white columns, AxV^{lo}; striped columns, AxV^{hi}.

RESULTS

PS Expression

Untreated CLL cells did not express detectable amounts of PS on their surface. Cells from six CLL patients were induced to undergo apoptosis by incubation with FAMP. Flow cytometric analysis at 24 h revealed three populations of AxV-positive cells, one being AxV^{neg} and two having distinctly different levels of AxV positivity, AxV^{hi} and AxV^{lo} (Fig. 1A). The marker between AxV^{neg} and AxV^{lo} cells was set according to unstained cells.

In other experiments, CLL cells were stained with AxV after FAMP exposure at seven different time points over a 24-h time period (Fig. 2). Over time, AxV^{lo} cells increased to approximately 30% whereas AxV^{neg} cells decreased and Ax^{hi} cells increased concomitantly, suggesting that AxV^{lo} cells represent a population of transition from AxV^{neg} to AxV^{hi}.

Membrane Integrity (PI)

PI positivity as a late marker of apoptosis was found only in a small fraction of cells in the compartment we defined as AxV^{hi}. PI positivity was not observed in AxV^{lo} or AxV^{neg} cells. The percentage of PI-positive cells increased over time as the membrane integrity of apoptotic cells was lost (data not shown).

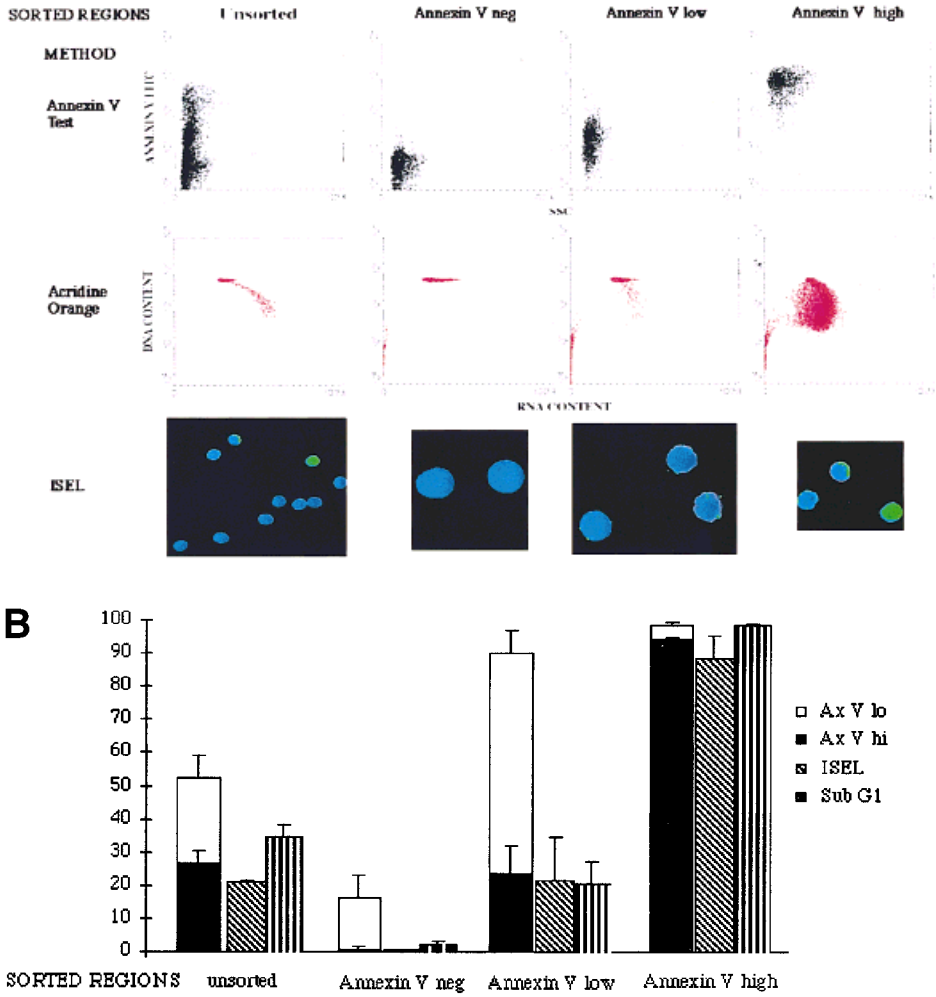

FIG. 3. Comparison of an unsorted region with three different sorted regions by three different assays in CLL cells treated with FAMP over 24 h. **(A)** Dot blots of AxV staining and sub G1 DNA content in lanes 1 and 2, respectively, and microscopic images of cells measured with ISEL. **(B)** Mean of two experiments . The y axis shows the percentage of positive cells of the different populations.

Comparison of PS Expression and DNA Fragmentation

In three experiments, cells were sorted according to the three assigned populations: AxV^{neg}, AxV^{lo}, and AxV^{hi}. They were then evaluated after restaining with AxV and acridine orange by flow cytometry and by ISEL on slides (Fig. 3A). AxV^{hi} and AxV^{neg} correlated with ISEL and the percentage of sub G1 cells as determined by DNA/RNA measurement by flow cytometry. Between 93 to 96% (mean, 94%; SEM, 0.6%) of cells were within the AxV^{hi} compartment after sorting for AxV^{hi} positivity. ISEL demonstrated 77–100% (mean, 88.6%; SEM, 6.6%) and acridine orange 98–99% (mean, 98.6%; SEM, 0.6%) of these cells to be apoptotic. Low percentages of AxV^{lo} cells (between 6% and 8%) and none of the AxV^{neg} cells were positive in the DNA fragmentation assays (Fig. 3B).

To obtain more detailed information on the dynamics of apoptotic cells in AxV^{lo}, we separated that population into four subpopulations (R1 through R4, Fig. 1B) in two experiments. Percentages of ISEL-positive cells increased from dim to bright in the four sorted populations, suggesting that increasing numbers of externalized PS molecules correspond with the progression of apoptosis within the AxV^{lo} population. Apart from cells with the characteristic homogeneous block-positive ISEL fluorescence, we observed cells with brightly fluorescing speckles (Fig. 5). Those cells increased in number in the different fractions from dim to bright (Fig. 4). These speckled cells were not seen in AxV^{neg} populations.

Cell Culture Experiments

Cells from each sorted population were placed back in RPMI and reanalyzed by AxV/PS staining after 24 h. Cells in the AxV^{hi} compartment expressed unmodified levels of PS, whereas cells in the AxV^{lo} compartment as well as the AxV^{neg} population increased their expression of PS. In the following 24 h of observation time, cells in the AxV^{lo} population progressed to AxV^{hi} and cells from the AxV^{neg} developed into AxV^{lo} and AxV^{hi} cells.

Expression of Bcl-2 Family Members in FACS-Sorted AxV Populations

The expression of Bcl-2 family members, which are among the most important regulators of apoptosis, was

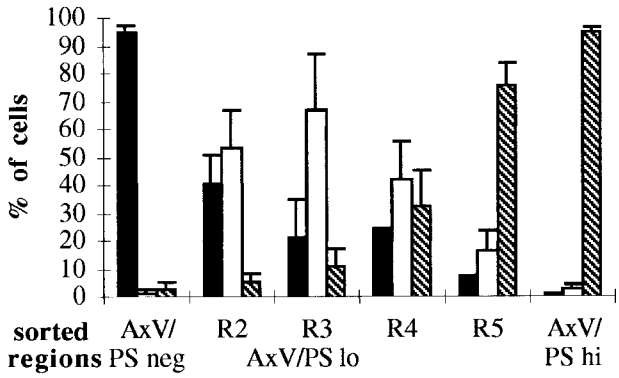

FIG. 4. Six sorted regions were reanalyzed by ISEL. Three different patterns of staining were found. ISEL-negative cells (black coulmns) decreased and ISEL-positive cells (homogeneously; striped columns) increased with increasing positivity for PS. Early apoptotic cells (speckled; white columns) increased early in the AxV^{lo} population but decreased again with the increase of ISEL-positive cells.

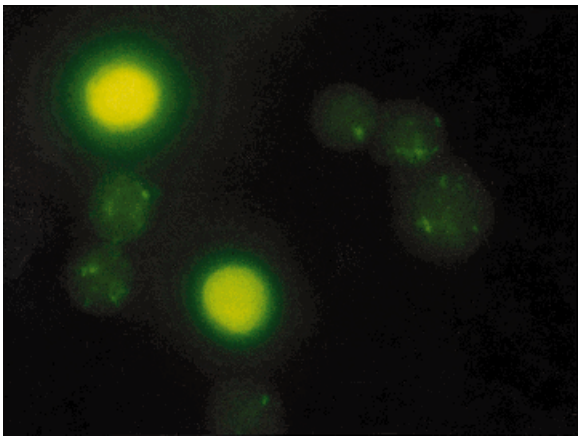

FIG. 5. Homogeneous block-positive cells and speckled cells.

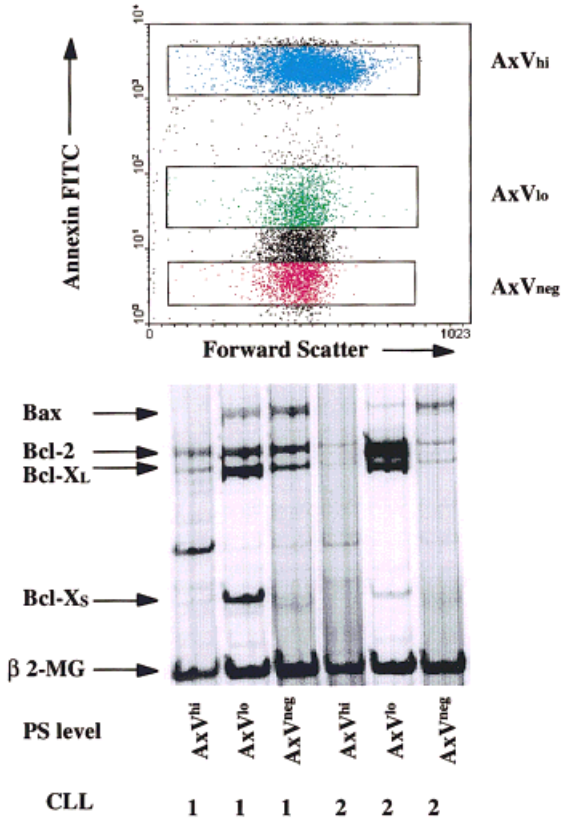

FIG. 6. Characteristic results of RNA RT-PCR for Bax, Bcl-2, Bcl-X_L, and BCL-X_S in two CLL samples (CLL 1, CLL 2). Bcl-2 and Bcl-X_L both are increased in the AxV^{lo} population compared to the AxV^{neg} population. The proapoptotic Bcl-X_S is only expressed in the AxV^{lo} population and Bax only in AxV^{neg} and AxV^{lo}. The dot plot (annexin FITC versus forward scatter) shows the sort regions used for CLL 1.

studied in FACS-sorted AxV populations using RT-PCR in five CLL samples treated with FAMP in vitro. In AxV^{neg} cells, Bcl-2, Bax, and low levels of Bcl-X_L mRNA were present (Fig. 6). Early apoptotic AxV^{lo} cells exhibited increased levels of Bcl-X_L and Bcl-2, whereas Bax decreased in some samples. Notably, pro-apoptotic Bcl-X_S was upregulated in early apoptotic AxV^{lo} cells. Nonapoptotic AxV^{neg} cells had no detectable pro-apoptotic Bcl-X_S and only low levels of Bcl-X_L and Bcl-2. Late apoptotic AxV^{hi} cells had no detectable levels of Bax and Bcl-X_S, and decreased expression of Bcl-2 and Bcl-X_L. In late apoptotic cells (AxV^{hi}) of both samples shown, a new band is detectable, probably representing fragments of Bcl-2 and Bcl-X_L, both of which are reported to be cleaved by activated caspase 3 (for review, see 15).

DISCUSSION

Apoptosis is a tightly regulated process that is crucial for maintaining homeostasis in a number of cell systems. It regularly results in internucleosomal DNA cleavage, which can be assessed by gel electrophoresis of DNA fragments "laddering." Earlier stages of apoptosis are not as well defined. Recently, it was observed that externalization of a particular membrane phospholipid, PS, to the outer cell membrane leaflet precedes DNA fragmentation during apoptosis (7–9,16). However, changes in PS externalization have been observed in many cell systems and can be related to cell-to-cell interactions, cell activation, and hemostasis as well as membrane fusion events (10). In establishing this assay for studies of apoptosis in hematopoietic malignancies (17), we noted different levels of PS expression and set out to establish a correlation between PS levels and apoptosis. In particular, it was not clear whether intermediate levels of PS were associated with apoptosis and whether low levels of PS expression were reversible and would therefore not be involved in the apoptotic process. Importantly, PS acts as a signal for macrophages to eliminate PS-positive cells, and is therefore an important component of the apoptotic pathway.

When we examined different levels of PS following exposure of CLL cells to FAMP, we found that PS/AxV^{neg} cell populations excluded PI and did not result in "sub G_1" peaks in DNA fragmentation assays, which was expected. Cells expressing high levels of PS/AxV had massive DNA fragmentation as assayed by ISEL and DNA flow cytometry. Interest-

ingly, we demonstrated that cells with low and increasing levels of PS/AxV are irrevocably committed to apoptosis as shown by the sorting of PS/AxV^{lo} cells and subsequent culture. These cells are initially negative for DNA fragmentation by conventional ISEL criteria. However, we observed a new pattern of ISEL positivity that we termed "speckled," which is indicative of early DNA fragmentation.

PI uptake in a cell is observed only after membrane damage and therefore serves as a marker for necrosis. When this phenomenon is observed in the course of PCD, it occurs only as a late apoptotic event and can then be referred to as secondary necrosis (16). This phenomenon probably can be studied in vitro only because late apoptotic cells in vivo are rapidly cleared by macrophages (4). PI-positive cells were found only in AxV^{hi}, suggesting that AxV^{hi} contains the latest apoptotic cells. ISEL and sub G1 both identify late apoptotic cells that are also AxV^{hi} but are positive only in a small fraction of AxV^{lo} cells. Flow cytometric analysis of CLL cells cultured in FAMP over time supports this view. The AxV^{lo} population, after increasing initially, reaches a steady state and decreases when most cells are apoptotic and the reservoir of viable cells declines, suggesting a transitional movement of apoptotic cells from dim to bright.

Using ISEL in situ, highly ISEL-positive, homogeneously stained cells from sorted, fractionated AxV^{hi} were found, whereas no end labeling was detected in AxV^{neg} in accordance with a previous report (9). In addition, we were able to demonstrate a time course in apoptosis correlated with the intensity in AxV staining, not only between AxV^{lo} and AxV^{hi} but also within the AxV^{lo} compartment itself. Percentages of ISEL-positive cells increased from low to high levels in four sorted populations. Apart from these few cells having the typical positive appearance, cells with brightly fluorescing speckles were observed. These speckled cells, increasing in number from dim to bright, were not seen in AxV^{neg}. In AxV^{hi}, speckles, if present, were quenched by the typical positive staining. Speckled cells of a similar kind have been described before (2). In accordance with our findings, they were found as an early apoptotic phenomenon prior to nuclear disintegration. Considering those data together with ours, speckled cells represent a potential means of detecting early apoptotic cells by applying TdT on slides. Utilizing flow cytometry, AxV is a more sensitive tool than TdT in detecting early apoptosis, as the speckled signal observed microscopically is too faint to be detected by flow cytometry.

In concordance with the results obtained by all three assays in AxV^{neg} to AxV^{hi} cells and considering the results of dissecting AxV^{lo} into four sorting regions of increasing brightness, we suggest that AxV^{lo} is associated with early apoptosis and that increasing levels indicate later stages of apoptosis.

The expression of Bcl-2 family members in the three different populations indicates that anti-apoptotic Bcl-2 and Bcl-X$_L$ mRNAs are expressed differently at different AxV levels. The transition from AxV^{neg} to AxV^{lo} was associated with increased expression of these anti-apoptotic genes. We have previously shown upregulation of Bcl-2 in leukemia patients treated with another related nucleoside analog, cytosine arabinoside (18) in vivo. This upregulation is associated with induction of pro-apoptotic Bcl-X$_S$, which can dimerize with both Bcl-X$_L$ and Bcl-2 (for review, see 15). The expression of Bax, which is expressed in untreated, AxV^{neg}, and early apoptotic cells, is surprisingly absent in late apoptotic cells. This indicates that Bax is not required at this late stage of apoptosis. Likewise, pro-apoptotic Bcl-X$_S$ is only detected in early apoptotic AxV^{lo} cells. This is consistent with the reported induction of massive apoptosis in cells expressing Bcl-X$_S$. The low levels of expression of Bcl-2 and Bcl-XL seen in the high apoptotic population may indicate lower levels of expression of these anti-apoptotic genes in cells that underwent apoptosis. The differential expression of Bcl-2 family members in different stages of apoptosis described here has not yet been reported.

It has been shown that protein expression of Bcl-2 and Bax does not change in the cause of induction of apoptosis through flavopiridol in CLL cells (19). Others have demonstrated the importance of these proteins in causing resistance to chlorambucil in B-CLL cells (20). Results presented here suggest a higher complexity of genes regulating cell death by the differential expression of more Bcl-2 family members at different stages of apoptosis.

In summary, the AxV^{hi} compartment represents the population that can be used for direct comparison with the DNA fragmentation assays ISEL and sub G1. Furthermore, AxV is more sensitive than these assays and enables the distinction between early and late apoptotic populations of cells in flow cytometry. For further study of the regulation of PCD, it will be possible to sort apoptotic cells with increasing levels of surface exposed PS, representing temporally discrete events following induction of apoptosis. The low or absent expression of Bcl-X$_S$ and Bax indicates that these pro-apoptotic genes are no longer needed when the apoptotic program is being executed.

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