Annexin V for Flow Cytometric Detection of Phosphatidylserine Expression on B Cells Undergoing Apoptosis


Apoptosis, or programmed cell death, is a general mechanism for removal of unwanted cells from the immune system. It is characterized by chromatin condensation, a reduction in cell volume, and endonuclease cleavage of DNA into oligonucleosomal length fragments. Apoptosis is also accompanied by a loss of membrane phospholipid asymmetry, resulting in the exposure of phosphatidylserine at the surface of the cell. Expression of phosphatidylserine at the cell surface plays an important role in the recognition and removal of apoptotic cells by macrophages. Here we describe a new method for the detection of apoptotic cells by flow cytometry, using the binding of fluorescein isothiocyanate-labeled annexin V to phosphatidylserine. When Burkitt lymphoma cell lines and freshly isolated germinal center B cells are cultured under apoptosis inducing conditions, all cells showing chromatin condensation strongly stain with annexin V, whereas normal cells are annexin V negative. Moreover, DNA fragmentation is only found in the annexin V-positive cells. The nonvital dye ethidium bromide was found to stain a subpopulation of the annexin V-positive apoptotic cells, increasing with time. Our results indicate that the phase in apoptosis that is characterized by chromatin condensation coincides with phosphatidylserine exposure. Importantly, it precedes membrane damage that might lead to release from the cells of enzymes that are harmful to the surrounding tissues. Annexin V may prove important in further unravelling the regulation of apoptosis.

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Rapid Communication

Materials and Methods

Reagents and monoclonal antibodies (MoAbs). Reagents used were Hoechst 33258 (25 ng/mL; Sigma Chemical Co, St Louis, MO) and ethidium bromide (EB), (5 ng/mL; BDH Chemicals Ltd, Poole, UK). Annexin V was prepared by cDNA recombinant techniques with plasmid pRH291, and purified as described before. The preparation was more than 99% pure. The only difference detected between recombinant and natural annexin V was an unblocked N-terminal alanine in recombinant annexin V, resulting in a slightly higher pI (4.9 v 4.8).

Annexin V FITC labeling. Annexin V was dialyzed against coupling buffer (50 mmol/L sodium borate/NaOH, pH 9.0, 150 mmol/L NaCl, and 1 mmol/L EDTA). Dialyzed annexin V, 50 μmol/L, was mixed with 50 μmol/L FITC isomer I (Sigma) and incubated for 2 hours at 37°C. The coupling reaction was then stopped by the addition of 100 mmol/L glycine. The mixture was firstly dialyzed against 50 mmol/L Tris/HCl, pH 8.0, 80 mmol/L NaCl, and 1 mmol/L EDTA and subsequently applied to a Mono Q column (Pharmacia,

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Uppsala, Sweden). The found proteins were eluted by an NaCl-gradient. The eluted peaks were analyzed for protein contents and adsorbance at 292 nm. The stoichiometric 1:1 complex was identified and used for the experiments. For detection of phosphatidylserine exposure on the cells, FITC-labeled annexin V was added at a final concentration of 2.5 µg/mL to cells incubated in HEPES buffer (10 mM HEPES/NaOH, pH 7.4, 150 mM/L NaCl, 5 mM/L KCl, 1 mM/L MgCl2, 1.8 mM/L CaCl2).

Cell lines and cell cultures. The BL cell lines Raji and Namalwa were cultured in RPMI 1640 medium with 25 mM/L HEPES buffer (GIBCO, Grand Island, NY) supplemented with 1 mM/L glutamine (GIBCO), penicillin/streptomycin (GIBCO), 10% fetal calf serum (FCS; GIBCO), and 10% hylocyte serum (Hylocyte Laboratories Inc, Logan, UT), at 37°C in a humid atmosphere and 5% CO2 in air. For apoptosis induction, the cells were washed several times in RPMI 1640 medium and then cultured in 6-well plates (Nunc; Intermed, Roskilde, Denmark) at 5 x 10^4 cells/mL in RPMI with 1% FCS as described.7

B-cell isolation. Germinal center B cells were isolated from tonsil tissue as described.22 In brief, freshly obtained tonsillar tissue was dissected free from surface epithelium, and finely minced into a cell suspension. Mononuclear cells were isolated by Ficoll Paque (Pharmacia) density gradient centrifugation. Monocytes were removed by plastic adherence for 1 hour at 37°C in 225-cm² cell culture flasks (Costar, Cambridge, MA). T cells were depleted using 2-aminoethylisothiouronium bromide modified sheep red blood cells. The purified B cells were then layered on a Percoll (Pharmacia) gradient, consisting of five density layers (1.085/1.077/1.067/1.056/1.043) and centrifuged for 15 minutes, 1,200g at 4°C. Cells at the 1.043/1.056 interface were harvested and cultured in a 24-well plate (Nunc; Intermed) at 1 x 10⁵ cells/mL in RPMI 1640 medium, supplemented with 10% FCS, 1 mM/L glutamine and penicillin/streptomycin at 37°C in a moist atmosphere with 5% CO2 in air.

Evaluation of apoptosis. At several time points, viability of the cells was measured by trypan blue exclusion. From the same well, cytocentrifuge preparations were made and stained with May-Grünwald/Giemsa for quantification of apoptosis on the basis of morphologic criteria, i.e., nuclear condensation and nuclear fragmentation (Fig 2). Until 36 hours of culture no changes in FACS profile were seen (not shown). However, after 34 hours of incubation a separate subpopulation was formed with a low forward scatter (FSC) and high side scatter (SSC) profile, which is a characteristic of apoptotic cells (Fig 1). The size of this subpopulation increased during the culture (Fig 1). Simultaneously an increasing proportion of the cells stained positive for annexin V. At 54 and 70 hours of culture both single annexin V-positive cells and annexin V/EB double-positive cells were found (Fig 1). At later time points (78 to 94 hours) all the apoptotic cells were double stained with annexin V and EB, possibly indicating that EB can only enter the apoptotic cell in a later stage when damage to the cell membrane has occurred. Cells in the “apoptotic” low FSC/high SSC subpopulation were all found to be annexin V-positive, whereas the cells with the normal FSC/SSC pattern were annexin V-negative (Fig 1).

The percentages of apoptosis as determined with May-Grünwald/Giemsa were found to correlate well with the percentages of annexin-positive cells (Fig 2). Importantly, the percentage of cells that stained with trypan blue were found to correspond to the percentage of EB-binding cells, and not with annexin V staining, which indicates that EB functions only as a nonvital stain in these experiments. Similar results were obtained for the BL cell line Raji (not shown). Culturing Namalwa at low serum concentration also resulted in formation of oligonucleosomal size DNA fragments, which were detected from 54 hours of incubation onward (not shown).

Detection of apoptosis with annexin V. To establish whether annexin V only stains apoptotic cells, Namalwa cells cultured at low serum concentration, were triple stained with Hoechst, annexin V, and EB, and analyzed by fluorescence microscopy. As shown in Fig 3, staining with Hoechst showed two different nuclear staining patterns. Part of the Namalwa cells showed a euhromatic staining pattern of the nucleus with Hoechst, which is similar to untreated non-apoptotic cells, whereas other cells showed a homogeneous intense staining of the nucleus or nuclear fragments that is characteristic for apoptotic cells with nuclear condensation.7 All cells with nuclear condensation were also stained with annexin V at the cell membrane, whereas none of the cells with an euchromatic nucleus were annexin V-positive (Fig 3). Occasionally a cell was found with only DNA condensation at the periphery of the nucleus (chromatin margination). These cells that represent the early stages of apoptosis7 were found to stain only weakly with annexin V. Thus, staining with annexin coincides with appearance of nuclear condensations in apoptotic cells. In this experiment only part of the
Fig 1. Flow cytometric analysis of apoptosis in Namalwa cells. Namalwa cells were cultured in 1% FCS for 54, 70, 78, or 94 hours. Cells were washed in annexin buffer and directly analyzed after addition of annexin V and EB. FSC (forward scatter)/SSC (side scatter) patterns (column 1) and annexin V/EB staining of un gated cells or of cells gated for low FSC/high SSC profile (gate 1) or high FSC/low SSC profile (gate 2) are shown in columns 2 to 4, respectively. Representative experiment out of three is shown.

Fig 2. Apoptosis and cell viability of Namalwa cells during culture at 1% FCS. After culturing Namalwa for 54, 70, 78, or 94 hours at 1% FSC, the percentage of trypan blue-positive cells (○), EB/annexin V double-positive cells (●), total annexin V-positive cells (comprising both the cells that only stain for annexin V plus the EB/annexin V double-positive cells) (■), and the percentage of cells showing nuclear condensations in the Giemsa staining (▲) was determined. Representative experiment out of three is shown.

In this report we describe a new method for the detection of B cells undergoing apoptosis by flow cytometry. The method uses the binding of FITC-labeled annexin V to phosphatidylserine, which is exposed on the surface of apoptotic cells but not on viable cells. The method allows for easy quantification of apoptosis on BL cell lines and germinal center B cells.

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Proper DNA fragmentation may lead to the disappearance of the oligonucleosomal fragments at this time point.

**DISCUSSION**

In this report we describe a new method for the detection of B cells undergoing apoptosis by flow cytometry. The method uses the binding of FITC-labeled annexin V to phosphatidylserine, which is exposed on the surface of apoptotic cells but not on viable cells. The method allows for easy quantification of apoptosis on BL cell lines and germinal center B cells.

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Fig 3. Triple staining fluorescence microscopy analysis of apoptosis in Namalwa cells. Namalwa cells were cultured for 70 hours in 1% FCS, after which the cells were stained with Hoechst, annexin V and EB. (A) Hoechst fluorescence showing, in blue, euchromatic nuclei and condensed nuclei with intense fluorescence. Occasionally EB-staining cells are seen (red fluorescence). (B) FITC fluorescence showing, in green, staining with annexin V at the membrane. Note that only cells with condensed nuclei are stained with annexin V. Original magnification ×560.

Without causing inflammation. During development of the T-cell repertoire in the thymus the nonresponsive and autoreactive T cells are removed through apoptosis. Similarly, during a secondary immune response, B cells in the germinal center are either selected to differentiate into memory B cells or plasma cells or they are deleted through apoptosis. Germinal center B cells spontaneously undergo apoptosis upon in vitro culture. BL cells, which are germinal center-cell derived, only enter apoptosis upon in vitro culture at low serum concentration. We have used these cell types to establish the applicability of annexin V for detection of apoptosis and have compared our results with previously described changes in cell morphology and DNA “ladder” formation.

Placing Namalwa, Raji, and germinal center B cells under apoptosis-inducing conditions resulted in nuclear condensation and staining with annexin V in a subpopulation of cells. Double staining with Hoechst showed that only the cells with nuclear condensation stain positive with annexin V. Moreover, the percentage of annexin V-positive cells correlates well with quantification of apoptosis by Giemsa staining. No intermediate staining with annexin V was found on the cells, which indicates that these apoptotic cells rapidly lose their membrane phospholipid asymmetry and expose phosphatidyl serine on the cell surface. At the early time points only part of the cells with condensed nuclei and annexin V staining were also positive with ethidium bromide. Thus, ethidium bromide only stains the cells in the later stages of apoptosis, probably when cell membranes have been damaged, which makes this reagent and other nonvital fluorescent dyes less suitable for detection of apoptosis.

Disruption of membrane integrity by nonionic detergents or during necrosis makes the cell interior accessible to annexin V. We observed that under these conditions cells are also stained with annexin V (not shown). However, they also stain positive for ethidium bromide and other nonvital dyes, which clearly contrasts with our observations on the early phases of apoptosis where the cells are ethidium bromide negative. Thus, staining with annexin V can be used as a specific marker for apoptosis in the early phase where the cell membrane is still intact. In a well-defined system such as the apoptosis of BL cells and germinal center B cells described here it also proves suitable for quantification of the later phases of apoptosis.

In the BL cells as well as in the germinal center B cells, internucleosomal cleavage, detected as DNA laddering, was found already at the earliest time points, when only a low percentage of the cells are annexin V-positive. At longer incubation periods the intensity of the oligonucleosomal bands decreases, whereas annexin V-positive cells and cells with condensed nuclei increase. Possibly, the cells that show
nuclear condensation and annexin V staining at a later time point in the culture have degraded their DNA into oligonucleosomal fragments at an earlier time point or, alternatively, they do not break down their DNA at all. Indeed, Sun et al. have recently shown with isolated nuclei that DNA fragmentation and chromatin condensation are differentially regulated processes. Still, sorting of apoptotic Namalwa cells on the FACS showed that the annexin V-positive subpopulation and not the negative cells showed nuclear condensation and internucleosomal DNA cleavage, indicating that the DNA fragmentation is limited to the morphologically defined apoptotic subpopulation.

The exposure of phosphatidyl serine on the surface of apoptotic cells has important implications. It triggers their specific recognition and removal by macrophages. The rapid phagocytosis of apoptotic cells may prevent the potential tissue damage resulting form the lysis of these cells in situ. Therefore, phospholipid asymmetry needs to be tightly regulated. Although the exact mechanism for maintaining the phospholipid asymmetry is still unclear, an adenosine triphosphate and magnesium-dependent membrane protein has been described that mediates the inward transport of negative-charged phospholipids. An additional mechanism for the maintenance of phospholipid asymmetry forms the association of phosphatidyl serine with membrane skeletal proteins. During apoptosis, changes in energy metabolism, bivalent cation concentrations, or cytoskeleton organization may be implicated in the regulation of phosphatidylserine exposure. Annexin V may prove important in defining further the regulation of apoptosis.

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