DETECTION OF APOPTOSIS BY 7-AMINO-ACTINOMYCIN D STAINING

MATERIALS:

1. 1 X PBS (PBSAz, 1 X PBS, e.g., Irvine Scientific, CA, containing 2% newborn calf serum and 0.1% sodium azide)

2. 7-Amino-actinomycin D (7-AAD, e.g., Cat #129935, EMD Millipore, MA)

3. Human AB serum, heat-inactivated (HAB, , e.g., from Innovative Research, MI)

4. Refrigerated centrifuge

Preparation of 7-AAD stock solution

Dissolve 7-AAD powder (1mg) first in 50 µL of absolute methanol, then add 950 µL of 1 X PBS. Final concentration is 1mg/mL. Keep solution at 4°C protected from light. Solution can be stored for at least up to 6 months.

METHOD:

Principle:

Apoptotic cells, probably due to a change in membrane permeability, take up some 7-AAD and become 7-AAD^{dim} compared to live cells which remain 7-AAD-. Late apoptotic or necrotic cells which have lost membrane integrity appear 7-AAD^{bright}.

Reference: Schmid I, Uittenbogaart CH, Keld B, and Giorgi JV: A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. J Immunol Meth 170: 145-157, 1994.

Staining for detection of apoptosis

1 x 10^6 PBSAz-washed cells from a single cell suspension are pelleted in a 12 X 75mm culture tube. The pellet is re-suspended in 1mL of 1 X PBSAz containing 20 µg/mL of 7-AAD and the suspension is mixed gently. After approximately 20 min of incubation at 4°C in the dark, the samples can be run on the flow cytometer in their staining solution. They should be kept cold during acquisition and should be run within 1 h after the addition of 7-AAD, otherwise cells should be fixed.

Note: it is possible to fix cells stained with 7-AAD for detection of apoptosis with a 1% formaldehyde solution containing 20 µg/mL of actinomycin D (AD, e.g., from Sigma-Aldrich, MO) for biohazard considerations and/or to preserve samples for later analysis on the flow cytometer. For procedure see attached protocol.

This staining procedure allows the correlation of apoptosis with single or dual-color phenotype and the use of FITC and/or PE conjugated monoclonal antibodies. Surface antigen staining is done according to standard methods before staining for apoptosis. Then, the apoptosis assay is performed as described above.

Direct Staining Procedure

1. Resuspend cell pellet first in 50 µL of HAB for approximately 1 min, then add 50 µL of PBSAz and the appropriate amount of fluorochrome-conjugated monoclonal antibody (mAb).

2. Vortex briefly and incubate for 15 min at 4°C in the dark.

3. Wash once with 2 mL of PBSAz by centrifugation at 250g for 5 minutes.

4. Proceed with staining for apoptosis as described above.

Indirect Staining Procedure

1-3 Process samples as above using a working dilution of unlabelled antibody.

4. Resuspend cell pellet first in 50 µL of HAB for approximately 1 min, then add 50 µL of a working dilution of the fluorochrome-conjugated second antibody.

5. Vortex briefly and incubate for 20 min at 4°C in the dark.

6. Wash once with 2 mls of PBSAz by centrifugation at 250g for 5 min.

7. Proceed with staining for apoptosis as described above.

Note: do <u>not</u> use HAB for staining of immunoglobulin chains. Whenever available, use a monoclonal antibody and/or a reagent directly conjugated to a fluorochrome to minimize unspecific binding. <u>Always</u> use isotypic controls of the same heavy chain class as your relevant antibody for determination of background staining.

Protocol for the use of actinomycin D (AD) on samples that were stained with 7-AAD for apoptosis and fixed in formaldehyde.

I. Materials:

1. Actinomycin D (AD, C_1, e.g., from Sigma-Aldrich, MO)

2. 1 X PBS

3. Sonicator

4. Formaldehyde solution (see protocol for preparation of 2% stock formaldehyde solution)

II. Preparation of AD stock solution (1mg/mL):

To 1mg of AD powder add:

50 µL of ice cold absolute ETOH, vortex

950 µL of 1 X PBS

Sonicate the resulting solution for 10 min at 4°C; keep the solution overnight in the refrigerator at 4°C, protected from light before using it.

Store solution at 4°C protected from light.

Working dilution is 20 µL /mL.

III. Method:

Cells are first incubated with 7-AAD for at least 20 min, then they are spun down once and immediately a 1% formaldehyde solution containing 20 µL /mL of AD (F/AD) is added to the cell pellet. Cells have to be stored in the cold protected from light and can be analyzed approximately 30 min after the addition of the F/AD solution. Cells are run on the flow cytometer in the F/AD solution. We have stored samples up to 3 days without any loss in the ability to discriminate early apoptotic from live cells and late apoptotic or necrotic cells.

INFORMATION AND REFERENCES ON DETECTION OF APOPTOTIC CELLS BY FLOW CYTOMETRY

Compared to the classic methods of DNA ladder formation by gel electrophoresis and of morphologic examination by electron microscopy for determination of apoptosis, flow cytometry permits rapid and quantitative measurements on apoptotic cells. Many different flow cytometric methods for the assessment of apoptosis in cells have been described (for a review see 1); most of these methods measure apoptotic changes in cells by staining with various DNA dyes, however, techniques using the terminal deoxynucleotidyl transferase or nick translation assays have also been developed (3). Some of these staining methods utilize unfixed cells (2,4,5,7,8,9,10,11). Due to the fragility of cells undergoing programmed cell death, rapid methods that maintain cells as close as possible to their natural state might be expected to provide the most reliable results. The current rapid 7-AAD staining method uses unfixed cells and thus permits the detection of changes in light scatter parameters and their correlation with other indicators of programmed cell death. However, one drawback of using any live staining method for measuring apoptosis is the variability of dye uptake in different cells and its possible change through certain treatment conditions. Furthermore, reagents which affect membrane permeability (e.g. calcium ionophores) cannot be used with this technique.

Many staining methods for flow cytometry use either fixed cells or treat cells with a hypotonic solution to permit DNA staining by non-vital dyes. The apoptotic cells with degraded DNA appear as cells with hypodiploid DNA content and are represented in so-called "sub-G1" peaks on DNA histograms (6,12). Telford *et. al.* (12) showed on murine thymocytes that many different

DNA dyes produce similar distributions of "sub-G1" peaks irrespective of their binding mode when the same cell fixation method was used. However, when we tried to apply the hypotonic PI method by Nicoletti *et al.* (6) to human thymocytes our results indicated that use of dissimilar sample preparation methods (e.g. live staining with HO342 vs. hypotonic PI) can lead to dramatic differences in the ability to detect apoptotic cells. Under certain conditions, the treatment of human thymocytes with hypotonic citrate solution containing detergent may not permit leakage of the low molecular weight DNA out of apoptotic cells; this leakage has been proposed as a pre-requisite for the formation of "sub-G1" peaks (1). In murine thymocytes, however, the hypotonic PI staining method produces a clear distinction between live and apoptotic cells (6).

Recently, it has been postulated that the stability and the kinetics of intermediate cell death stages may determine the resistance of a particular cell type to DNA degradation. Consequently, the visibility of "sub-G1" peaks by flow cytometry and also the formation of characteristic DNA ladders on agarose gels might be a function of individual cellular homeostasis. The absence of a "sub-G1" peak on a DNA histogram should not constitute proof of no apoptosis (1). Conversely, it has also been stated that the mere appearance of a hypodiploid DNA peak should not be taken as definite evidence for the presence of an apoptotic cell population without other supporting information (7). Flow cytometric findings in a particular cell type exposed to a certain stimulus must therefore be always verified with other non-flow cytometric methods. Recently, rapid flow cytometric staining methods that use Annexin V for detection of phosphatidylserine exposure on the cell surface as a marker of apoptosis have become commercially available. For this staining method it is essential to add a dead cell discrimination dye like propidium iodide or 7-amino-actinomycin D to the stained cells, because late apoptotic or necrotic cells also express phosphatidylserine and have to be distinguished from the early apoptotic cells by fluorescence (13). Newer flow cytometric assays measure Caspase-3 activity, an early marker of cells undergoing apoptosis and kits are commercially available (14).

Please keep in mind that not all methods for detection of apoptosis in cells may be equally sensitive and techniques must be assessed critically with respect to their applicability to a particular cell type or system.

References

The following reference list is by no means complete, but should provide a starting point for background information related to the measurement of apoptosis by flow cytometry. It includes early seminal publications that describe the first methods as well as two recent reviews that give a historical perspective and summarize more recently developed techniques.

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