MEASURING APOPTOSIS AND NECROSIS BY HOECHST STAINING AND DEAD CELL DISCRIMINATION

MATERIALS:

1. 1 X PBS (PBS-BSA, without sodium azide + 2% bovine serum albumin)

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

3. Hoechst 33342 (HO342, e.g., Cat# H3570 LifeTechnologies, Grand Island, NY)

4. Propidium iodide (PI, e.g., Cat# 537059 EMD Millipore, MA)

5. 7-Amino-actinomycin D (7-AAD, e.g., Cat #129935, EMD Millipore, MA)

6. 37°C water bath, centrifuge

7. Bucket with ice

Preparation of stock solutions of HO342, PI, and 7-AAD

HO342

Dilute 10mg/mL HO342 stock solution to 1mg/mL in distilled H2O. Keep solution at 4°C protected from light. Solution can be stored for at least up to 6 months.

PI

Dissolve PI powder at a concentration of 1mg/mL in 1 X PBS. Keep solution at 4°C protected from light. Solution can be stored for at least up to 6 months.

7-AAD

Dissolve 7-AAD powder (1mg) first in 50 µL of absolute methanol or DMSO, 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, due to a change in membrane permeability, show an increased up-take of the vital dye HO342 compared to live cells. PI or 7-AAD is added to discriminate late apoptotic or necrotic cells which have lost membrane integrity from early apoptotic cells which still have intact membranes by dye exclusion.

References:

Ormerod MG, Collins MKL, Rodriguez-Tarduchy G, Robertson D: Apoptosis in Interleukin-3 dependent haemopoietic cells: quantification by two flow cytometric methods. J Immunol Meth 153:57-65, 1992.

Schmid I, Uittenbogaart CU, Giorgi JV: A sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. Cytometry 15:12-20, 1994.

Staining for detection of apoptosis

1 x 10^6 PBS-BSA-washed cells from a single cell suspension are pelleted in a 12 X 75mm culture tube. The pellet is re-suspended in 1ml of PBS-BSA pre-warmed to 37°C and the suspension is mixed gently. Then, 1 µL of HO342 stock solution is added and the mixture is immediately vortexed briefly. The suspension is incubated for exactly 7 min at 37°C, then placed on ice, and 1 µL of PI stock solution or 1 µL of 7-AAD stock solution is added. After approximately 10 min the samples can be run on the flow cytometer. They should be kept cold during acquisition and should be run within 1 hour after the addition of the DNA dyes.

This staining procedure allows the correlation of apoptosis with single or dual-color phenotype. 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. Re-suspend cell pellet first in 50 µL of HAB for approximately 1 min, add 50 µL of PBS-BSA 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 PBS-BSA by centrifugation at 250g for 5 min.

4. Resuspend samples in 1 mL of PBS-BSA pre-warmed to 37°C.

5. 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, 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 mL of PBS-BSA by centrifugation at 250g for 5 min.

7. Resuspend samples in 1 mL of PBS-BSA pre-warmed to 37°C.

8. Proceed with staining for apoptosis as described above.

Note: do not 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. Always use isotypic controls of the same heavy chain class as your relevant antibody for determination of background staining. While FITC- and AlexaFluor488-labeled mAbs are compatible with PI can be used, PE and other fluorochromes that overlap with PI require the use of 7-AAD.

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.

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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