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The Use of Flow Cytometry for Concomitant Detection of Apoptosis and Cell Cycle Analysis

Introduction

Two distinct modes of cell death, apoptosis and necrosis, can be distinguished on the basis of differences in morphological, biochemical, and molecular changes occurring in the dying cells (1). Cells undergoing apoptosis display a characteristic pattern of structural changes in the nucleus and cytoplasm, including rapid blebbing of the plasma membrane and nuclear disintegration (1). Extensive damage to chromatin and cleavage of DNA into oligonucleosomal-length fragments both occur during apoptosis (1,2). This DNA fragmentation is considered to be the key biochemical event of apoptosis (1,2).

Several flow cytometric methods for identifying cells undergoing DNA fragmentation have been described recently. These include DNA content analysis and *in situ* labeling of DNA fragments with tracer-dUTP. The former is based on the accumulation of ethanol-fixed apoptotic cells in the sub-G₀/G₁ peak of the DNA content histogram as a result of loss of DNA fragments out of the cells and because of a reduced DNA "stainability" (3,4). The latter uses exogenous terminal deoxynucleotidyl transferase (TdT) to label *in situ* the DNA strand breaks with a tracer-dUTP (5,6).

Recent observations have revealed a profound regulatory interrelationship between apoptosis and the cell cycle (7). The investigation of this relationship ideally requires techniques that permit concomitant apoptosis detection and cell cycle analysis at a single-cell level.

Two flow cytometric techniques are usually used to investigate the cell cycle: DNA quantification to identify the cell cycle position (8) and detection of bromodeoxyuridine (BrdU) incorporation to reveal cells going through the S phase (9). In this investigation, we

report the development of flow cytometric techniques that permit concomitant detection of apoptosis and cellular DNA content or BrdU content analysis by adapting the apoptosis detection protocol of the Boehringer Mannheim *In Situ* Cell Death Detection Kit, Fluorescein.

Materials and Methods

Cell culture

Thymocytes prepared from 6 week old mice (C57 black x CBA) were cultured in RPMI 1640 medium (GIBCO, Gent, Belgium) supplemented with 10% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (GIBCO). To induce apoptotic cell death, 0.5 µM of ionomycin (Sigma, Bornem, Belgium) was added to the culture medium as described by Kizaki *et al.* (10).

Cell fixation

The cells were harvested and washed with phosphate-buffered saline (PBS) containing 10% FCS. Cell pellets were resuspended in 250 µl PBS, followed by 250 µl PBS containing 2% paraformaldehyde. After an incubation period of 15 minutes at 4°C, cells were washed, resuspended in 5 ml of ice cold 70% ethanol, and incubated overnight at -20°C.

DNA fragmentation detection

After two washes of the fixed cells with PBS, the TUNEL reaction was carried out as described in the Boehringer Mannheim *In Situ* Cell Death Detection Kit, Fluorescein, package insert. Briefly, cells were incubated in a moist chamber (37°C) with the TUNEL reaction mix as stated in the suppliers pack insert. Control samples were treated identically except that the TUNEL reaction medium lacked the TdT enzyme. After an incuba-

tion period of 1 hour, cells were washed twice with PBS containing 10% FCS and 0.1% Triton® X-100. At this point, cell samples were either resuspended in PBS and analyzed by flow cytometry, or they were processed further for measurement of DNA or BrdU content.

DNA quantification

Fixed cells processed for DNA fragmentation detection were resuspended in PBS containing 200 µg/ml ribonuclease and 5 µg/ml propidium iodide (PI) and then incubated for 30 minutes at room temperature as described by Gorczyca *et al.* (5). Cells were then analyzed for green (FITC, indicating DNA fragmentation detection) and red (PI, allowing DNA quantification) fluorescence by flow cytometry. DNA content analysis obtained with this protocol was compared with the technique described by Vindeløv *et al.* (8).

BrdU detection

The relative number of cells incorporating BrdU was estimated as previously described by Gratzner (9). For this assay, cell culture medium was supplemented with 10 µM BrdU. Fixed cells processed for DNA fragmentation detection were washed with PBS, resuspended in 2 ml of 2 M HCl containing 0.5% (v/v) Triton® X-100, and incubated at room temperature for 30 min. Subsequently, cells were centrifuged and resuspended in 2 ml of 0.1 M Na₂B₄O₇, pH 8.5, to neutralize acidity. After another centrifugation, cell pellets were resuspended in 200 µl of PBS containing 0.5% (v/v) Tween® 20 and 1% (w/v) BSA. The cells were spun down, and anti-BrdU mouse monoclonal antibody that had been prediluted in PBS containing 0.5% (v/v) Tween® 20 and 1% (w/v) BSA was added. The cells were incubated in the dark for 30 minutes at

37°C and washed with PBS containing 0.5% (v/v) Tween 20 and 1% (w/v) BSA. Pellets were resuspended in PBS containing 0.5% (v/v) Tween 20, 1% (w/v) BSA, and R-phycoerythrin-conjugated F(ab')₂ goat anti-mouse IgG (H+L chains) (Dako, Gentbrugge, Belgium) diluted to a predetermined working concentration 100-fold. After a 30-minute incubation at 37°C, cells were washed and resuspended in PBS. Cell samples were then analyzed for green (FITC, indicating DNA fragmentation) and orange (R-phycoerythrin, indicating BrdU detection) fluorescence by flow cytometry.

Results and Discussion

Adaptation of the cell fixation procedure

Frequently, cell cycle analysis techniques require cell fixation in ethanol for permeabilization (8,9). Unfortunately, this procedure is associated with the loss of low molecular weight DNA from fixed cells and, therefore, decreases the incorporation of FITC-dUTP and the DNA content of apoptotic cells (5).

To ensure both cell permeabilization and cross-linking of the low molecular weight DNA to other cellular constituents (5), the cells were successively fixed in paraformaldehyde and ethanol (a two-step fixation procedure). The first fixative prevents the loss of DNA fragments from apoptotic ethanol-fixed cells by cross-linking the low molecular weight DNA to other cellular constituents (5); therefore, it allows the correlation between DNA content of fixed cells and cell cycle position.

To determine the compatibility of the two-step fixation procedure with the detection of apoptosis using the Boehringer Mannheim *In Situ* Cell Death Detection Kit, Fluorescein, the percentage of apoptotic cells was compared to that obtained following the manufacturer's protocol. After a 2- or 12-hour incubation with the apoptosis inducer ionomycin (0.5 μM), cells were harvested, fixed according to the two-step cell fixation procedure, and processed further for analysis.

Figures 1A and 1B show the percentages of apoptotic cells determined by the cell fixation procedure described in the commercial kit. Twelve hours after induction of apoptosis, we used the cell fixation procedure described in the commercial kit to obtain a percentage of

apoptotic cells (29%) (Figure 1B) similar to that obtained with the two-step fixation procedure (28%). We, therefore, used the two-step fixation procedure for the next two experiments.

Concomitant detection of apoptosis and DNA content analysis

To determine the compatibility of apoptosis detection and DNA content quantification, we compared the results of DNA content analysis obtained using our protocol with those obtained following the technique described by Vindeløv *et al.* (8). After a 2- or 12-hour incubation with 5 μM ionomycin, cells were harvested, fixed, and processed further for DNA fragmentation detection and DNA content analysis.

The cell cycle distribution obtained following our protocol was comparable to that obtained with the Vindeløv technique (8). In terms of percentage of cells in the different phases of the cell cycle, comparable results were obtained for the two protocols (data not shown). However, the coefficient of variation for the G₀/G₁ peak was higher with our protocol (5.14%) than with the Vindeløv technique (3.67%).

To check whether DNA content analysis did influence apoptosis detection, we compared the percentages of apoptotic cells obtained following our protocol with those obtained with the protocol for apoptosis detection described in the Boehringer Mannheim *In Situ* Cell Death Detection Kit, Fluorescein, package insert. Again, 12 hours after induction of apoptosis, using the procedure described in the commercial kit, we obtained a percentage of apoptotic cells (29%) similar to that obtained with the protocol used to detect apoptosis and analyze the DNA content (30%) (Figures 1B and 1D). These observations indicated that apoptosis detection and DNA content analysis were compatible and, therefore, could be used for concomitant apoptosis detection and DNA content analysis.

The results presented in Figures 1C and 1D clearly showed that apoptotic cells featured a DNA content near to that of cells in G₀/G₁ or early S phases. Since S phase cells incorporate BrdU but G₀/G₁ phase cells do not (9), we established a protocol that analyzes DNA fragmentation and BrdU content concomitantly.

Concomitant detection of apoptosis and BrdU content analysis

To determine whether apoptosis detection was compatible with BrdU detection, we compared the results of BrdU content analysis obtained following our protocol with those obtained following the technique described by Gratzner (9).

After a 2- or 12-hour incubation with 0.5 μM ionomycin, cells were harvested, fixed, and processed further for DNA fragmentation detection and BrdU content analysis. In 12 hour cultures, the percentage of BrdU-positive cells (21%) (Figure 1F) was similar to the percentage obtained following the procedure described by Gratzner (22%) (reference 9). These results indicated that BrdU detection was not altered by the protocol used to detect apoptosis.

We also checked whether BrdU detection could influence apoptosis detection. When compared with the results of the apoptosis detection procedure described in the Boehringer Mannheim *In Situ* Cell Death Detection Kit, Fluorescein (29%, Figure 1B), the percentage of cells detected as apoptotic (27%, Figure 1F) was not affected.

These observations indicated that apoptosis detection and BrdU detection were also compatible and, therefore, could be used for concomitant detection of apoptosis and BrdU content analysis. Using this technique, we can determine both the percentage of G₀/G₁ phase and the percentage of early S phase apoptotic cells. Figure 1F shows the percentage of apoptotic cells positive for BrdU (6%), indicating that these cells went through the S phase. It also shows that 21% of cells were positive for DNA fragmentation but negative for BrdU incorporation, corresponding to the G₀/G₁ fraction of apoptotic cells.

Conclusion

This report describes the concomitant detection of apoptosis and cell cycle analysis techniques. These techniques include (i) the two-step cell fixation procedure, (ii) concomitant detection of apoptosis and DNA content analysis, and (iii) concomitant detection of apoptosis and BrdU content analysis.

The two-step cell fixation procedure has numerous advantages. By preventing the loss of DNA fragments from ethanol-

fixed apoptotic cells (5), it allows the correlation of DNA content of fixed cells and cell cycle position. This cell fixation procedure is compatible with concomitant detection of an intracellular antigen (e.g., viral protein) and DNA fragmentation (data not shown). Finally, cells fixed following this procedure can be stored at -20°C for up to 3 days before further processing (5).

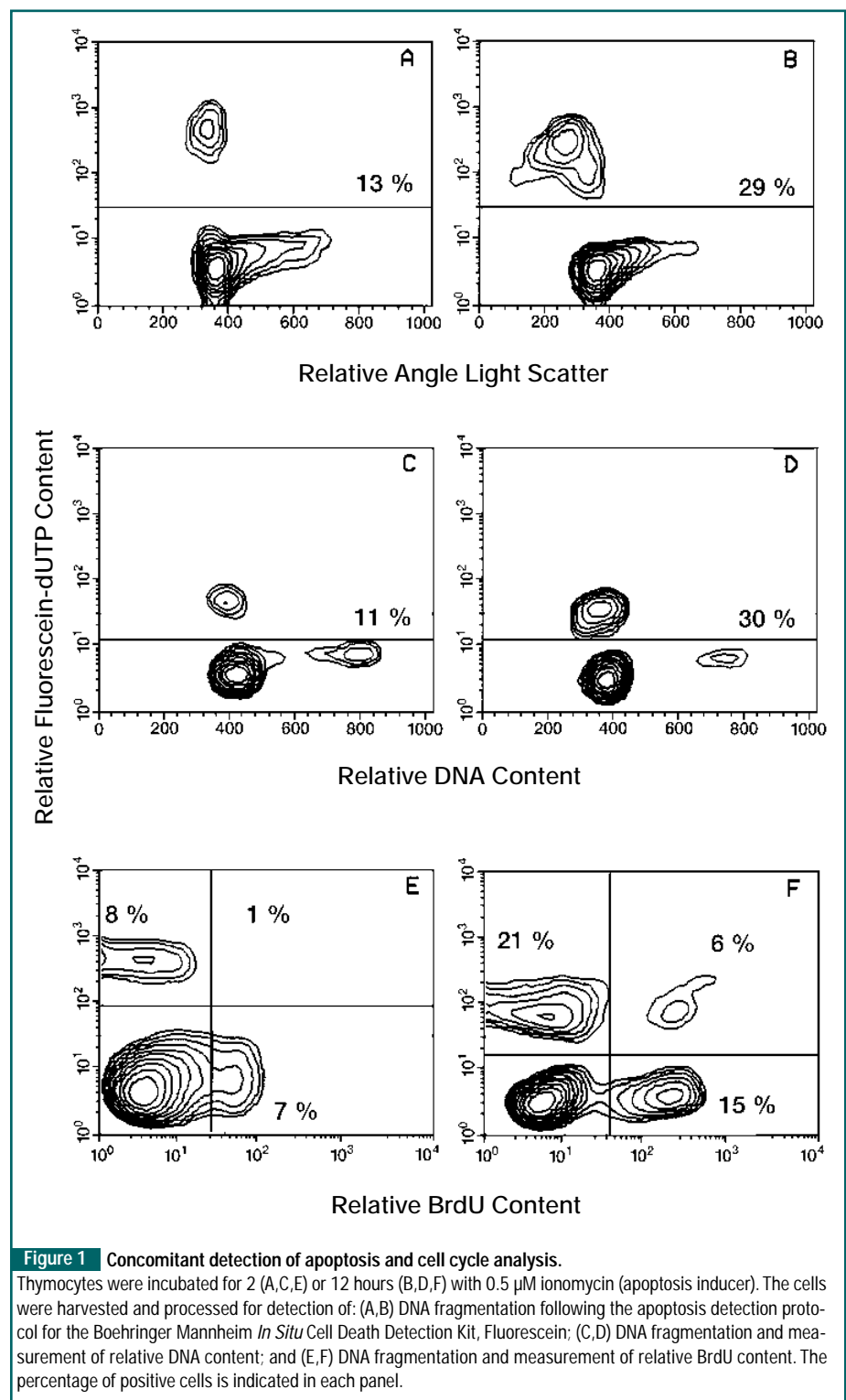
Concomitant detection of apoptosis and DNA content or BrdU content analysis permits, at a single-cell level, detection of apoptosis and determination of cell cycle phase by flow cytometry. The cell cycle position is determined by concomitant detection of apoptosis and DNA content analysis. The differentiation between apoptotic S phase cells from G_0/G_1 and G_2/M phase cells is made possible by a concomitant detection of apoptosis and BrdU content analysis.

Detection of apoptosis and cell cycle analysis will be very helpful in the understanding of the precise relationship between apoptotic cell death induction and the cell cycle. Moreover, these techniques will also be applicable in assays requiring both detection of an antigen (BrdU, viral protein) and apoptotic cell death.

Acknowledgments

The authors would like to thank Dr. J. Lyaku (Liège, Belgium) for helpful comments on the manuscript. We thank M. Loncar, L. Karelle-Bui Thi, J.-P. Georin, and A. Brichaud for excellent technical assistance. A. Vanderplassen is a Senior Research Assistant of the Fonds National Belge de la Recherche Scientifique (FN.R.S.). E. Hanon is a Research Assistant of the FN.R.S.

Product	Cat. No.	Pack Size
<i>In Situ</i> Cell Death Detection Kit, Fluorescein	1684 795	1 kit (50 tests)
Anti-Bromodeoxyuridine, formalin grade	1170 376	50 μg



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