**Original Articles**

**New Method for the Analysis of Cell Cycle–Specific Apoptosis**

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**Background:** In this study, a new method for the analysis of cell cycle specificity of apoptosis was designed by using a modified annexin V and propidium iodide (API) method.

**Methods:** Cells of the human promyelocytic HL-60 line treated with camptothecin (CPT) or ultraviolet light (UV) were labeled with fluorescein isothiocyanate–conjugated annexin V and prefixed with 1% methanol-free formaldehyde on ice, and their DNA was stained stoichiometrically with propidium iodide in the presence of digitonin. Cellular green and red fluorescences were measured by flow cytometry.

**Results:** Cell cycle specificity of apoptosis obtained by the API method and those analyzed for the presence of DNA strand breaks by using terminal deoxynucleotidyl transferase (TdT) assay were similar: CPT- or UV-induced apoptosis preferentially in S- or G1-phase cells, respectively. When the internucleosomal DNA degradation was prevented by the serine protease inhibitor N-tosyl-L-phenylalanine chloromethyl ketone, apoptotic cells could not be detected by the TdT assay but were identified by the API method.

**Conclusions:** The API method, similar to the TdT assay, accurately detects the cell cycle phase specificity of apoptosis. Also, the API method appears to detect earlier stages of apoptosis than the TdT assay.

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**Key terms:** apoptosis; cell cycle; annexin V; terminal deoxynucleotidyl transferase

Apoptosis (programmed cell death) is controlled by a variety of genes and functional proteins whose transcription and activity are timely induced in response to stress that triggers this mode of cell death (1–12). Apoptotic events are highly coordinated with each other, and errors in execution of apoptosis may lead to severe diseases (1–16). Therefore, analysis of the incidence of apoptosis and monitoring of particular events of apoptosis are of importance not only in basic laboratory studies but also in the clinical setting.

During the past decade, different methods for analysis of apoptosis have been developed (1,2,17). The methods using flow cytometry, by virtue of providing accurate estimates of apoptotic index in large cell populations and by offering a possibility of multiparameter analysis, are the most widely used in detection of apoptosis (18–22). Because most anticancer drugs and many other agents that induce apoptosis target cell cycle–specific events, analysis of apoptosis with respect to the cell cycle position is of particular interest. This can be accomplished by multiparameter analysis of the flow cytometric data when cellular DNA content, the parameter that defines the cell cycle position, is measured as one of the cell attributes (10–12,14,23–29).

In this study we combined an assay of annexin V binding to identify apoptotic cells with the stoichiometric staining of cellular DNA using propidium iodide (PI) to define the cell cycle position. This method (API) was compared with analysis of DNA strand breaks by using an exogenous terminal deoxynucleotidyl transferase (TdT; or terminal dUTP nick end labeling [TUNEL]) assay. The results showed that the API method is more rapid and has
an advantage over the TUNEL method, which detects apoptosis in the instances when DNA fragmentation stops at the 300- to 50-kb section, in not progressing into inter-nucleosomal sections.

MATERIALS AND METHODS

Cell Culture and Induction of Apoptosis

HL-60 cells, purchased from American Type Culture Collection (Rockville, MD), were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco) and 2 mM l-glutamine (Sigma Chemical Co., St. Louis, MO). The experiments were performed on cells during their exponential phase of growth, as described elsewhere (23).

Fresh stock solutions of camptothecin (CPT; Sigma) were prepared at a drug concentration of 200 μg/ml in dimethyl sulfoxide, and fresh stock solutions of N-hydroxy-L-phenylalanine chloromethyl ketone (TPCK; Sigma) were prepared at a concentration of 50 mM in ethanol. Further dilutions were made by addition of RPMI-1640 medium. The cells were incubated at 37°C in the presence of 0.15 μM CPT or 0.15 μM CPT plus 10 μM TPCK, respectively, for up to 4 h. Control cultures were treated with an equivalent volume of dimethyl sulfoxide and ethanol in RPMI. In addition, the flasks containing cell cultures during exponential growth were placed directly on the glass surface of a mini-transilluminator (0.375 W/cm²; Bio-Rad, Hercules, CA) illuminated with ultraviolet (UV) light for 40 s (150 kJ/m²) and subsequently incubated at 37°C for 4 h. Then the cells were harvested, sedimented (200 g, 5 min), resuspended in phosphate buffered saline (PBS; Sigma), and divided into two portions. One portion was used for annexin V labeling, and the other was fixed for the DNA strand break assay and agarose gel electrophoresis. One flask of exponentially growing cells was treated with 0.15 μM CPT, and cell aliquots from that culture were harvested at 30-min intervals for up to 4 h for the detection of apoptotic rate.

DNA Gel Electrophoresis

Untreated or drug- or UV-treated cells were collected by centrifugation and fixed with 75% ethanol pre-cooled to 0–4°C by adding ethanol drop-wise under constant agitation. The cells were maintained in ethanol at −20°C for at least 24 h. The cells were then centrifuged at 200g for 5 min; the ethanol was thoroughly removed; the cell pellets, in 1.5-ml Eppendorf tubes, were resuspended in 40 μl of 0.2 M phosphate citrate buffer, pH 7.8, at room temperature for at least 30 min; after centrifugation at 800g for 5 min, the supernatant was used for agarose gel electrophoresis as described previously (21). The degree of DNA fragmentation was also assayed by using pulsed filed gel electrophoresis according to the procedure as described by Filipski et al (30).

Annexin V and PI Labeling (API)

Common procedure for detection of total apoptosis. Aliquots of freshly collected cells suspended in PBS were centrifuged (200g, 5 min) and resuspended in binding buffer, pH 7.4, containing 10 mM Hepes (Sigma), 140 mM NaCl, and 2.5 mM CaCl₂ to have approximately 10⁶ cells/ml. Five microliters of fluorescein isothiocyanate (FITC) plus annexin V (Pharmingen, San Diego, CA) and 10 μl of PI (Sigma) at a concentration of 50 μg/ml in PBS was then added, and the cells were incubated for 20 min at room temperature in the dark, as described previously (31,32).

Procedure for the analysis of cell cycle–specific apoptosis (API method in brief). Five microliters of FITC-annexin V was added to 100 μl of freshly collected cells suspended in binding buffer at a density of 10⁶ cells/ml. The cells were placed at room temperature in the dark for 20 min, rinsed in binding buffer, resuspended in 1 ml of 1% methanol-free formaldehyde in binding buffer for 30 min on ice, rinsed twice, resuspended in 0.5 ml of PI solution containing 50 μg/ml PI, 0.1% RNase A (Sigma), 500 μg/ml digitonin (Sigma), 10 mM PIPES (Sigma), 2 mM CaCl₂, and 0.1 M NaCl, placed at room temperature for 1 h in the dark, and analyzed by flow cytometry for the cell cycle specificity of apoptotic cells. Untreated HL-60 cells served as the negative control.

DNA Strand Break Labeling by TdT

The harvested cells were prefixed in suspension in 1% methanol-free formaldehyde (Polysciences, Warrington, PA) in PBS, pH 7.4, on ice for 15 min, as described previously (20), rinsed and postfixed in 75% cold ethanol, and placed at −20°C. After fixation, the cells were treated twice with PBS and then resuspended in 50 μl of reaction buffer solution containing 0.2 M potassium cacodylate (Sigma), 2.5 mM Tris-HCl (pH 6.6), 2.5 mM CoCl₂ (Sigma), 0.25 mg/ml bovine serum albumin (Sigma), 5 U of terminal deoxynucleotidyl transferase (Boehringer Mannheim Biochemical, Indianapolis, IN), and 0.5 mM of biotinylated dUTP (Boehringer Mannheim). The cells were incubated in this solution at 37°C for 45 min, rinsed in PBS, and resuspended in 100 μl of a solution containing four times concentrated saline sodium citrate buffer, 25 μg/ml FITC-conjugated avidin (Boehringer Mannheim), 0.1% Triton X-100 (Sigma), and 5% bovine serum albumin, incubated in this solution for 30 min at room temperature in the dark, rinsed in PBS containing 0.1% Triton X-100, and resuspended in 1 ml of PBS containing 5 μg/ml of PI and 0.1% RNase (Sigma). Untreated HL-60 cells served as the negative control.

Flow Cytometry

The fluorescence of cells stained with FITC-conjugated avidin and PI in the TdT assay and the fluorescence of cells stained with FITC-conjugated annexin V and PI in API assay were measured on a FACSort Flow Cytometer (Becton Dickinson, San Jose, CA). CELLQuest software was used for acquisition and analysis of data.

RESULTS

Quantitative Analysis of Apoptotic Cells

Table 1 presents the results of apoptotic rates of HL-60 cells measured by common staining with FITC-annexin V.
and the API method. The exponentially growing cultures of HL-60 cell were treated by UV, CPT, and CPT plus TPCK. The total apoptotic index of each sample was measured 10 times by using the three approaches described above. The results obtained by the API method were similar to those obtained by the standard FITC-annexin V assay (t-test, $P > 0.05$).

Apoptotic indices of the samples treated with UV and CPT were $10.7 \pm 1.6$ and $19.5 \pm 1.9$, respectively, when estimated by the TdT assay, which is significantly lower than the API method ($17.3 \pm 1.8$ vs. $34.7 \pm 1.9$) and standard annexin V staining (t-test, $P < 0.01$). The results of samples treated with CPT plus TPCK were negative when measured by the TdT assay (Fig. 1).

Figure 2 shows the relation between CPT-inducing time and apoptotic index detected by the API method and the TdT assay. HL-60 cells, which were in the exponential phase of growth, were incubated at $37^\circ C$ in the presence of $0.15 \mu M$ CPT for different time intervals; every 30 min, a 10-ml aliquot of cell suspension was removed; and the apoptotic cells were detected by the API method and TdT assay. The apoptotic cells were evident after 1.5 h when detected by the API method, and the apoptotic cells were not evident until 2.5 h when detected by the TdT assay. The data indicated that the API method detects earlier stages of apoptosis than does the TdT assay.

Table 1

<table>
<thead>
<tr>
<th>Detecting methods</th>
<th>Apoptotic index (%) after different treatment</th>
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<tr>
<td></td>
<td>UV</td>
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<tr>
<td>Standard FITC + annexin V staining</td>
<td>18.1 $\pm$ 1.5</td>
</tr>
<tr>
<td>API method</td>
<td>17.3 $\pm$ 1.8</td>
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*API, annexin V plus propidium iodide; CPT, camptothecin; FITC, fluorescein isothiocyanate; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; UV, ultraviolet.

**DNA Agarose Gel Electrophoresis**

DNA extracted from the fixed HL-60 cells by phosphate citrate buffer, as described previously (14), was subjected to agarose gel electrophoresis. DNA in the gels was visualized under UV light after staining with $5 \mu g/ml$ of ethidium bromide (Sigma). As evident in Figure 3, the DNA extract samples from cultures treated with UV light and CPT demonstrated a clear ladder pattern on gel electrophoresis, and the ladder intensity with CPT was distinctly higher than that of UV light, whereas no DNA was detected in the gels from samples representing the untreated (control) cells or the cells treated with CPT plus TPCK. Analysis of DNA size by pulsed gel electrophoresis indicated the presence of DNA fragments of at least 50 kb in extracts of cells treated with CPT combined with TPCK, similar to that described by Hara et al. (33) (not shown).

**Cell Cycle Specificity of Apoptosis**

After inducing apoptosis of HL-60 cells by UV, CPT, or CPT plus TPCK, the cell cycle distribution of apoptotic and nonapoptotic cells was analyzed with the API method and the TdT assay, respectively. Figure 4 presents the results as density contour plots representative of the cells treated with UV light, CPT, or CPT combined with TPCK. It is evident that the cells exposed to UV preferentially underwent apoptosis in G1 phase, those treated with CPT underwent apoptosis in S phase, and those treated with UV and CPT plus TPCK underwent apoptosis in both G1 and S phases.

Fig. 1. Apoptotic indices of HL-60 cells detected by the API method and the TdT assay. The cells were treated with UV light, 0.15 $\mu M$ CPT, and 0.15 $\mu M$ CPT plus 10 $\mu M$ TPCK.

Fig. 2. Relation between inducing time and apoptotic index. HL-60 cells were induced with 0.15 $\mu M$ CPT and harvested every 30 min. The apoptotic cells were identified by the API method and the TdT assay.
CPT plus TPCK showed no cell cycle phase specificity. The mean fluorescence intensities of the FITC-positive (apoptotic) cells in G₁ and S phase were 20- and 16.4-fold higher, respectively, than that of the negative cells when detected with the API method. In contrast, the mean fluorescence intensities of FITC-positive cells in G₁ and S phases was only 13- and 11.6-fold higher, respectively, than that of the negative cells when detected with the TdT assay. In addition, in the contour plot induced by CPT, there were no cells in the S phase of the negative control region when detected with the API method, whereas there were some residual S-phase cells in the negative control region when detected by the TdT assay. This result suggested that the sensitivity of the TdT assay is lower than that of the API method.

After induction of apoptosis by CPT plus TPCK, the frequency of apoptotic cells detected by the API method was the same as that induced by CPT only, but the TdT assay did not detect apoptotic cells.

**DISCUSSION**

Quantitative detection and cell cycle specificity analysis of apoptosis are very important for the study of the molecular mechanism of cell death and cell cycle progression. Flow cytometry offers the advantages of rapid, sensitive, and multiparameter analysis of asynchronous cell populations and has been used widely for the investigation of the biological processes associated with cell death (1,2,17–22).

The approach based on DNA strand break labeling in the assay employing TdT has appeared to be the most specific in terms of positive identification of apoptotic cells until now (1,4,22,24). In this way, cellular DNA content of not only nonapoptotic cells but also apoptotic cells is measured; the method offers a unique possibility to analyze the cell cycle position and DNA ploidy of apoptotic cells.

It has been shown that loss of phospholipid asymmetry leading to exposure of phosphatidylserine on the outside of the plasma membrane is an early event of apoptosis (31,32). The principle of standard FITC-annexin V staining used for the detection of apoptosis is that annexin V can preferentially conjugate negatively charged phosphatidylserine, and apoptotic cells in different stages have different abilities to resist PI. By this way, we can quantitate apoptotic cells and distinguish them from necrotic cells. The standard assay, however, does not provide information of the cell cycle specificity of apoptosis. We observed that methanol-free formaldehyde can fix cells without
dissociation of annexin V, and the optimal concentration of formaldehyde is 1%. In addition, formaldehyde crosslinks DNA and, hence, prevents leakage of the fragmented DNA from apoptotic cells. This allows one to identify the cell cycle position of apoptotic cells. In the API method, digitonin is used to increase cell permeability (34) after fixation in formaldehyde. The presence of calcium ions is essential to maintain attachment of annexin V to phosphatidylserine during fixation and subsequent staining with PI. We observed that the nonionic detergent that otherwise is used to permeabilize the plasma membrane causes dissociation of annexin V, which manifests as decreased fluorescence.

TPCK in this study was used to prevent internucleosomal DNA fragmentation; in its presence after induction of apoptosis, DNA cleavage stops at 300- to 50-kb sections and does not progress further (35). Thus, because of paucity of DNA strand breaks, the TdT assay was unable to identify apoptotic cells in the TPCK-treated cultures. Therefore, it appears that TdT assay may fail to detect early apoptotic cells and atypical apoptotic cells, in which DNA fragmentation did not progress to internucleosomal DNA sections.

As the present data showed, the API and standard FITC-annexin V binding methods produce consistently higher results than the TUNEL method. One explanation for the discrepancy may be that the “time window” of the annexin V binding assay is wider, i.e., the cells become first annexin V positive and sometime later TUNEL positive.

In conclusion, similar to the TdT assay, the API method accurately detects the cell cycle–phase specificity of apoptosis. However, compared with the TdT assay, it detects earlier stages of apoptosis and thus has a wider time window for identification of apoptotic cells. Thus, the apoptotic index by the API method is higher that the TdT assay. Because the TdT assay fails to detect apoptosis when DNA degradation is not progressing to internucleosomal DNA sections, as happens in some cases of “atypical apoptosis,” the API method is preferred in these instances. Furthermore, compared with the TdT assay, the API method has fewer steps, is more rapid, and appears to be more sensitive. However, the API method also has limitations. Because the key point of API is the conjugation of annexin V and phosphatidyl serine, the factors that injure plasma membrane can lead to pseudo-positive results.

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