

Supravital Exposure to Propidium Iodide Identifies Apoptosis on Adherent Cells

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Background: Several studies indicate that plasma membrane changes during apoptosis are a general phenomenon. Among the flow cytometric methods to measure apoptosis, the Annexin V assay that detects the membrane exposure of phosphatidylserine (PS) is one of the most commonly used. However, the various treatments used for the detachment of adherent cells generally interfere with the binding of Annexin V to membrane PS, making apoptosis measurement a technical problem.

Materials and Methods: Apoptosis of different cell lines was investigated by fluorescence microscopy and multiple flow assays designed to assess loss of membrane integrity, translocation of PS, DNA fragmentation, and light scatter changes.

Results and Conclusions: We show that supravital propidium iodide (PI) assay stains adherent apoptotic cells, allowing flow cytometric quantification. Moreover, supravital exposure to PI without prior permeabilization identifies apoptotic cells as well as Annexin V and permits the simultaneous surface staining by FITC- and PE-conjugated monoclonal antibodies. As in the case of necrotic or permeabilized cells, fluorescence microscopy has revealed that PI staining of apoptotic cells is localized in the nucleus. This suggests that the binding of PI to the DNA/RNA structures is stable enough to withstand the trypsinization and/or washing procedures necessary to detach adherent cells. Cytometry 44:57–64, 2001.

Key terms: adherent cells; propidium iodide; apoptosis; necrosis; surface antigens

Apoptosis, by counteracting cell proliferation, plays a fundamental role in the development of tissues and organs, in the regulation of immunological response, and in the elimination of senescent cells (1–3). The loss of regulation of the apoptotic pathways is often associated with tumor growth, and radiotherapy and chemotherapy usually result in apoptotic tumor cell death. Apoptosis can be induced by chemical and physical agents in tumor and/or normal cells. Drugs that can produce apoptotic cell death in tumor cell lines are potentially useful in chemotherapy.

The qualitative and quantitative evaluation of apoptosis in a cell population has been approached by several techniques. Optical and electronic microscopy, which detects apoptotic morphological features, was the first method to be used, and is still used, to verify the presence of apoptotic cells in a sample (2). Gel electrophoresis, which detects genomic DNA fragmentation, was considered the hallmark of programmed cell death (1). The technique is used to qualitatively discriminate between different kinds of apoptosis (with or without nucleosomal DNA fragmentation). Finally, flow cytometry has allowed the quantitative measurement of apoptotic cells (4–6).

Programmed cell death is usually associated with DNA fragmentation (1,7–13). However, examples of apoptosis without DNA fragmentation (14–18) or DNA cleavage without apoptosis (19) have been described, inducing the reconsideration of morphological techniques as a golden standard for the identification of apoptosis. Apoptotic morphological features observed by microscopy are still important to define, at least qualitatively, the phenomenon. Several flow cytometric assays have been developed to monitor and measure early signs of apoptosis in clinical trials as well as in basic science studies (4–6,15,20–24).

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Besides the partial loss of cleaved DNA, another important parameter that flow cytometry evaluates, is cell membrane alteration. One of the most common flow cytometric methods utilizes Annexin V to detect early membrane changes in apoptotic cells (5,6,23,25,26). In previous studies, we demonstrated that the supravital exposure to propidium iodide (PI) of unfixed cells in suspension led to the simultaneous identification of living (PI negative), apoptotic (PI dim) and necrotic (PI bright) cells (15,22). In the present report, we compare this easy flow cytometric assay, based on supravital exposure to PI, with Annexin V assay and/or hypodiploid DNA content. Notably, supravital exposure to PI allows the simultaneous detection of living, apoptotic, and necrotic cells. It is a reliable assay even when exposure of phosphatidylserine (PS) residues does not indicate the presence of apoptosis, as in the case of adherent cells after detachment (23,26).

MATERIALS AND METHODS

Reagents

Stock solution included 1 mg/ml PI (Calbiochem, La Jolla, CA) in phosphate-buffered saline (PBS). Annexin V-FITC was obtained from Clonetech Laboratories (Palo Alto, CA). Anti-CD7-FITC (Coulter-Immunotech, Marseille, France), anti-CD71-FITC, and anti-CD3-PE (Becton Dickinson, Immunocytometry Systems, San Jose, CA) monoclonal antibodies (mAb) were added contemporaneously with PI for 30 min at room temperature for the simultaneous detection of apoptosis and immunophenotype.

Cell Culture

Jurkat, Raji, and HL-60 human leukemic cell lines were grown in suspension. SK-N-MC human neuroblastoma (27) and MCF-7 human breast carcinoma cell lines were cultured adherent in RPMI 1640 in a 5% CO_2 humidified incubator at 37°C. Cell line cultures were supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 20 mM Hepes (pH 7.5). For the MCF-7 cell line, 1 mM insulin was also added (Sigma, St. Louis, MO). Peripheral blood cells from different donors were collected in K_3EDTA tubes. Peripheral blood mononuclear cells (PBMC) from voluntary donors were obtained by Ficoll-Hypaque gradient centrifugation. PBMC were incubated 30 min at 37°C in plastic Petri dishes to eliminate adherent cells. Peripheral blood lymphocytes (PBL) and cell lines were used for apoptosis experiments.

Induction of Apoptosis

Apoptosis was induced by UVB irradiation on PBL, Raji, HL-60, SK-N-MC, and MCF-7. Cells in 25-ml culture flasks (1×10^6/ml) were exposed for 20 min to a 302-nm UVB transilluminator source. They were incubated successively at 37°C, 5% CO_2 for 4 or 7 h. The Jurkat cell line was treated at 37°C, 5% CO_2 for 5 or 6 h with 1 μg/ml of recombinant tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL; 28).

Detection of Apoptosis

Apoptotic cells were detected by four flow cytometric methods and by light or fluorescence microscopy.

Flow Cytometry. The flow analysis was performed by a FACScan or FACStar plus (Becton Dickinson) equipped with an argon ion laser tuned at 488 nm wavelength. Red, orange, and green fluorescence intensities were collected in log scale, whereas forward scatter (FSC) and side scatter (SSC) were collected in linear scale. The emission filters used were 530 ± 30 nm band pass for FITC (FL1), 585 ± 42 nm band pass for PE (FL2), and 650 nm long pass for PI (FL3). Electronic compensation was used to eliminate bleed through fluorescence. For tricolor analysis, the following settings were used: 560, 450, and 540 mV on photomultiplier tubes for FL1, FL2, and FL3, respectively. Appropriate values of electronic compensation were adjusted between each fluorochrome: FL1, 0.6% FL2; FL2, 23.6% FL1; FL2, 38.4% FL3; and FL3, 13.0% FL2. Data analysis was performed with Lysys II or CellQuest software (Becton Dickinson).

For supravital PI staining, nonpermeabilized cells were incubated during the last 30 min in the dark with 50 μg/ml PI. The suspended cell lines were washed with PBS and then analyzed by flow cytometry (or light microscopy). For simultaneous detection of apoptosis and immunophenotype, 2×10^5 cells in 200 μl of PBS were incubated with 50 μg/ml PI and the indicated mAb for 20 min in the dark at 4 °C. The cells were then washed and analyzed by flow cytometry. SK-N-MC and MCF-7 adherent cell lines were incubated during the last 30 min with 50 μg/ml PI. The adherent cells were rinsed twice with PBS to remove PI and harvested by standard trypsinization (0.5 mg/ml trypsin and 0.2 mg/ml EDTA in PBS; Sigma). Detached cells were collected with the supernatants, pelleted by centrifugation, and washed with PBS to remove PI. Detached and adherent cells were finally pooled and resuspended in PBS before flow cytometric (or light microscopic) analysis. Apoptotic and necrotic cells were detected as a PI^{dim} and PI^{bright} cluster, respectively. For simultaneous detection of apoptosis and immunophenotype on adherent cell lines, 2×10^5 adherent cells in 200 μl of PBS were incubated with the indicated mAb for 20 min in the dark at 4°C, washed, and analyzed by flow cytometry.

For PI staining on ethanol-fixed cells, cells were fixed in 70% cold ethanol for at least 30 min at 4°C. They were washed in PBS and incubated for 30 min at 37°C with 20 μg/ml PI, 100 μg/ml RNase A type XII (Sigma), 25 mM Na_3citrate, 0.05 M Na_2HPO_4, and 0.1% Triton X-100, pH 7.8 (citrate buffer). Apoptotic cells were detected on a PI histogram of ethanol-treated cells as a hypodiploid peak (20).

For Annexin V staining, cells were washed and resuspended in 200 μl of 1 × binding buffer (Clonetech Laboratories). Staining and analysis were performed following the manufacturer's instructions. For simultaneous detection of supravital PI and Annexin V staining, after induction of apoptosis, nonpermeabilized cells were incubated

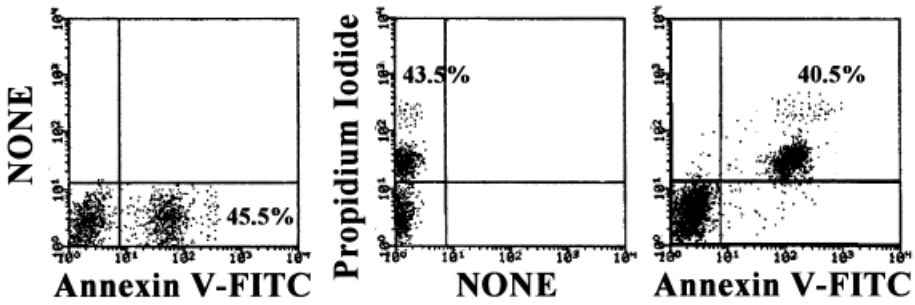

FIG. 1. Supravital PI and Annexin V assays identify the same apoptotic cells. Jurkat cells were cultured in medium for 6 h with recombinant TRAIL (1 μg/ml). Cells were separately or simultaneously stained with FITC-Annexin V and PI (see Materials and Methods) and analyzed by flow cytometry. Percentages of apoptotic cells are indicated. Data are representative of three experiments.

for 30 min at room temperature in the dark with 50 μg/ml PI. The cells were then washed with PBS, resuspended in 200 μl of 1 × binding buffer, and stained with 5 μl of FITC-Annexin V for 10 min before flow cytometric analysis.

At each experiment, samples were also subjected to FSC/SSC analysis. It is well known that, due to cell shrinkage, apoptotic cells generally have decreased FSC. For this reason, FSC usually represents a gross indicator of morphological changes that occur on cells undergoing apoptosis.

Light/fluorescence microscopy. After apoptosis induction and supravital PI staining (described above), cells were washed and spun on slides. For microscopic observations, a Vanox microscope (Olympus Italia, Milan, Italy) equipped with a mercury lamp was used with a BP545 excitation filter. For viewing both PI-negative viable cells (which tend to exclude PI) and PI-positive apoptotic cells, simultaneous application of transmitted light phase-contrast and fluorescence microscopy was used.

In some experiments, cells were spun on coverslips, fixed in cold methanol for 5 min, stained with Giemsa (Sigma), diluted 1:20 in PBS for 30 min at room temperature, rinsed twice with PBS, and observed at light microscopy.

RESULTS

Detection of Apoptosis by Combined Supravital PI and Annexin V Assays

The supravital PI staining and Annexin assays detected similar percentages of apoptosis in Jurkat cells treated for 6 h with 1 μg/ml of TRAIL (Fig.1). To ascertain whether both techniques identify the same populations, a simultaneous detection of apoptosis by supravital PI and Annexin V assays was performed. Virtually all PI-positive cells were positive for FITC-Annexin V; moreover, all viable cells, which excluded PI, were not stained by FITC-Annexin V (Fig.1, right panel). Similar results were obtained with UV-treated PBL (not shown). These findings strongly indicate that both techniques identify the same apoptotic populations.

Simultaneous Detection of Apoptosis and Immunophenotype

The cultures of Jurkat cells incubated for 5 h with or without TRAIL (1 μg/ml) were stained with anti-CD7-FITC mAb and PI as indicated in Materials and Methods. Dot plots of FITC (X axis) versus PI (Y axis) fluorescence are represented in Figure 2A (upper panels). Figure 2 shows that supravital PI staining allows the simultaneous identification of apoptotic cells and cell surface staining with FITC-conjugated mAbs. At the same time point, the hypodiploid peak produced by apoptotic cells in ethanol-fixed samples was also present on treated cells (Fig. 2A, bottom panels). Furthermore, numerous apoptotic cells and a few apoptotic fragments were already evident at light microscopy examination (Fig. 2B). To ascertain the possibility to perform three-color analysis, PBLs were stained with anti-CD3-PE mAb and PI (Fig. 2C). As shown in the Figure 2C, using the appropriate compensation settings (see Materials and Methods), supravital PI assay permits the simultaneous surface staining with PE-conjugated mAbs. Early apoptosis (PI^{dim}) and cells in secondary necrosis (PI^{bright}) are also distinguished. Thus, our technique allows the accurate quantification of apoptotic and necrotic cells together with their phenotype.

Detection of Apoptosis by Supravital PI Assay on Adherent Cells

To verify that the supravital PI staining, differently from Annexin V, could detect apoptosis in adherent cells, PI was added 30 min before cell harvesting to UV-treated and untreated SK-N-MC neuroblastoma cells. Before detachment, cells were rinsed with PBS; supernatants and cells detached after trypsinization were centrifuged and pooled together. Supravital PI staining detected apoptosis on these cells after only 4 h of incubation. At this time point, PI^{dim} apoptotic cells were detectable with high (50 μg/ml), but not with low (5 μg/ml), PI concentration (Fig.3A), indicating that a high concentration of PI is necessary to stain apoptotic cells. Although a clear hypodiploid peak was not present yet (not shown), changes in light scatter characteristics associated with apoptotic trigger were detectable with both PI concentrations in UV-

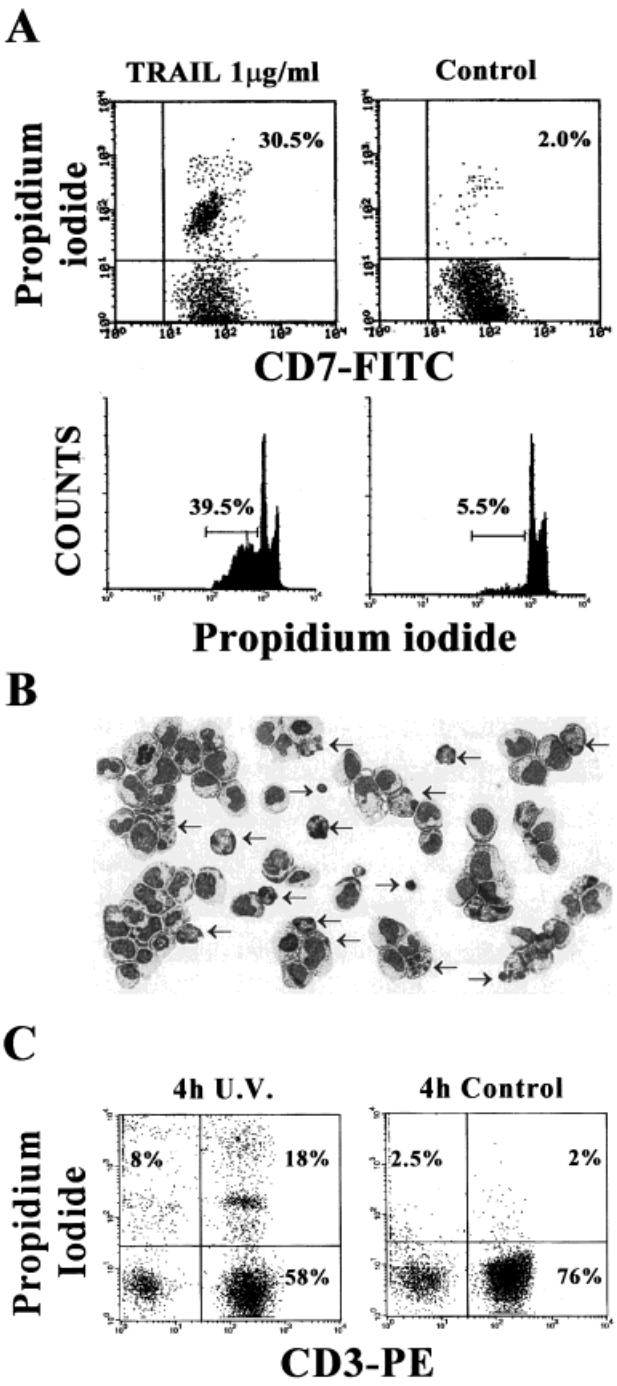

FIG. 2. Apoptosis and cell surface phenotype detection by supravital PI assay. **A,B:** Jurkat cells were incubated for 5 h with or without TRAIL (1 μg/ml). Cells were labeled with anti-CD7-FITC mAb and PI (A, upper panels) or they were ethanol fixed and then stained with PI (A, bottom panels), as indicated in Materials and Methods. Jurkat cells treated for 5 h with TRAIL were also stained with Giemsa and observed by light microscopy (B). Apoptotic fragments and apoptotic cells are indicated by arrows (×600). **C:** PBLs were treated for 20 min with UV and control samples were incubated at 37°C, 5% CO_2 for 4 h. Cells were stained with anti-CD3-PE mAb and PI, as described in Materials and Methods. Percentages of apoptotic cells are indicated. Data are representative of at least three experiments.

treated cells (Fig.3A, upper panels and not shown), indicating that apoptosis did not depend on PI concentration. At 7 h of incubation after UV treatment, comparison between supravital PI exposure and hypodiploid peak detected by PI on ethanol-treated cells confirmed that the supravital PI assay was a reliable and early marker for detection of apoptosis in adherent cells (Fig. 3B). Changes in light scatter characteristics detected at 7 h were more evident than those at 4 h and, surprisingly, were characterized by an increase in FSC (Fig.3B, upper panels). To confirm that the supravital PI assay worked in combination with surface antigen detection on different apoptotic models of adherent cells, SK-N-MC and MCF-7 adherent cell lines were stained with anti-CD71-FITC mAb and PI. Figure 3C shows that supravital PI staining allows the simultaneous identification of apoptotic cells on adherent cell lines and cell surface staining by FITC-conjugated mAbs independently on antigen density. In agreement with previous reports (23,26), but at variance with suspended cells, necrotic (PI^{bright}) cells were not detected in adherent cells (Fig. 3).

Detection of Apoptosis by Fluorescence Microscopy

To independently prove that PI^{dim} cells were apoptotic cells, UV-treated cell lines were analyzed by fluorescence microscopy after supravital PI exposure. Figure 4 shows examples of merged images of PI fluorescence and cell morphology of HL-60, SK-N-MC, and Raji cell lines. Viable cells are represented by faint peripheral staining generated by simultaneous application of fluorescence and transmitted light phase-contrast microscopy. PI labeling of the nucleus was observed in cells showing typical nuclear fragmentation and chromatin condensation.

DISCUSSION

Programmed cell death is an active process that leads to deep nuclear alterations, cytoplasmatic shrinkage, progressive membrane permeability, and decrease of surface antigen density (4–6). Ionizing radiation, enzymatic inhibitors, growth factor withdrawal, and death receptor triggering are the most common apoptotic stimuli, some of which are used against tumor cells.

Among the activators of death receptors, we used TRAIL, a member of the TNF ligand family. Previous data had suggested that TRAIL selectively killed cancer cells and it was proposed as an anticancer molecule (29). Nevertheless, it has to be taken into account that our recent data indicate that it is able to induce a rapid cell death not only on tumors (28), but also on differentiating cells (30).

Many flow cytometric protocols that target different cellular compartments have been described to detect apoptotic cells. Most of them are focused on the evaluation of changes of light scatter parameters, indirectly associated with morphological alterations (4,5,20); low incorporation of PI due to the loss of fragmented DNA after fixation (4,5,31); PI incorporation due to early alterations of the cell membrane on unfixed cells (6,15,22); and changes of mitochondrial membrane potential (5,32–34).

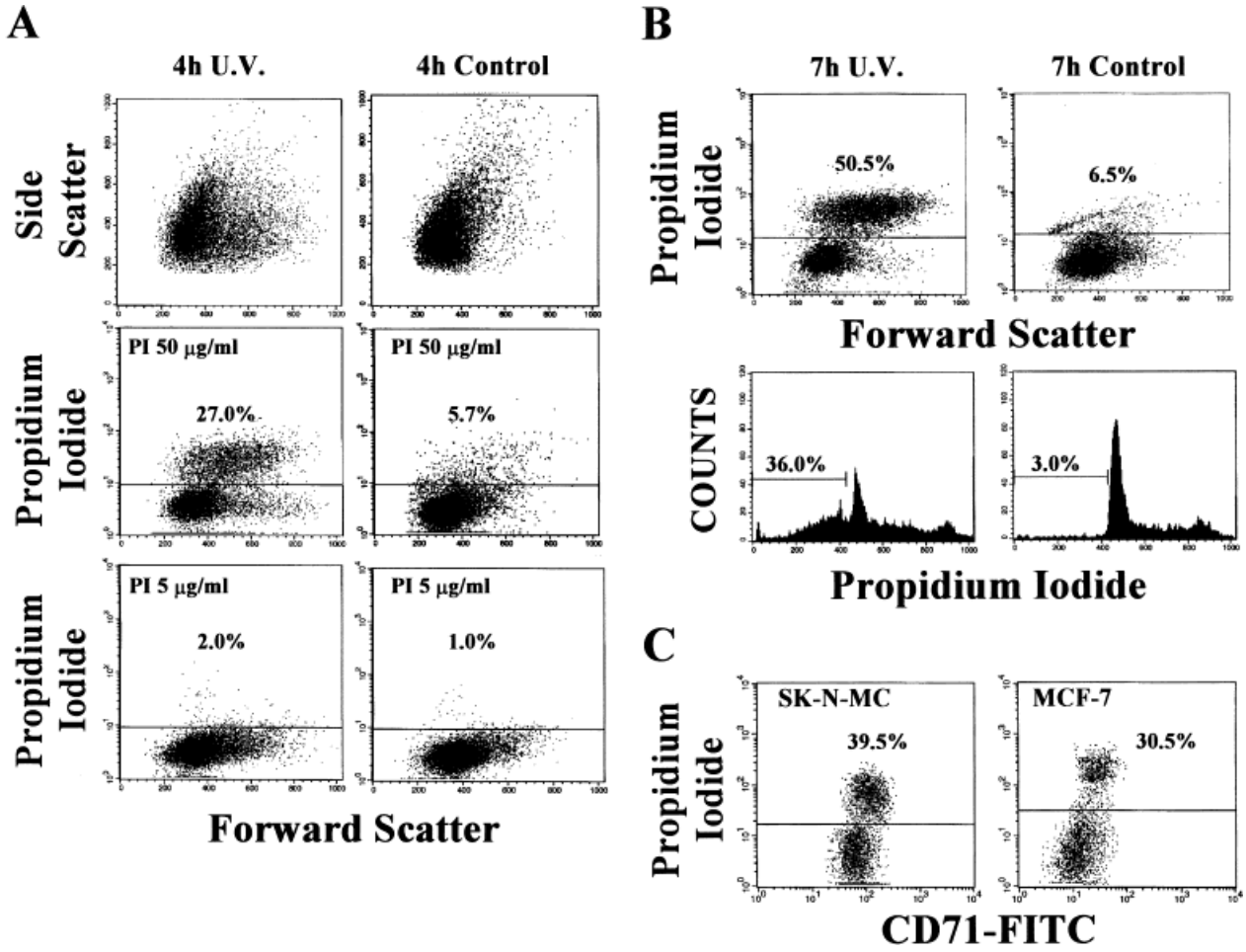

FIG. 3. Detection of apoptosis on adherent cells. UV-treated and untreated neuroblastoma adherent cells were incubated for 4 h **(A)** or 7 h **(B)**. Scatter plots (A, upper panels, PI 5 μg/ml) and PI fluorescence versus FSC plots of cells stained with 50 μg/ml (A, middle panels) or 5 μg/ml (A, bottom panels) and percentages of PI-positive cells. Comparison of apoptotic cell percentages detected by supravital PI assay and by PI staining in ethanol-treated cells is shown in PI fluorescence versus FSC plots (B, upper panels) and PI histograms (B, bottom panels), respectively. UV-treated neuroblastoma (left panels) and MCF-7 (right panels) adherent cells were incubated for 7 h and then stained with PI and anti-CD71-FITC mAb **(C)**. Data are representative of at least three experiments.

In the present study, the apoptosis of different cell lines was investigated by multiple flow cytometric assays designed to assess the loss of membrane integrity (supravital PI staining); translocation of PS (Annexin V staining); DNA fragmentation (PI staining after ethanol treatment); and light scatter changes (FSC/SSC analysis). Our data confirm that changes in DNA content, detected by PI incorporation, and changes in light scatter characteristics, which are typical of apoptotic cells, appear independently of each other and usually late during the apoptotic development. This seems to be related to the apoptotic model. In the Jurkat cell line induced to apoptosis by TRAIL, the hypodiploid peak appears earlier than the scatter modifications (not shown). The opposite is true in the case of the neuroblastoma cell line SK-N-MC treated with UV. On the other hand, supravital PI staining, as well as Annexin V, recognizes early apoptotic cells. As an example, at 4 h of incubation after UV treatment of SK-N-MC cells, staining of apoptotic cells by supravital PI exposure is evident before the hypodiploid peak appears and when a few scatter changes are present. The percentages of apoptotic SK-N-MC cells detected by the hypodiploid peak are reproducibly lower than those measured by the supravital PI assay, as expected, but the opposite is true for TRAIL-treated Jurkat cells. Because membrane alterations are usually considered an early event if compared with DNA fragmentation, the percentages of apoptosis based on hypodiploid peak should be constantly lower than those obtained by supravital PI staining. This unexpected result can be explained by the rapid nuclear fragmentation induced by TRAIL that, after the fixation/permeabilization procedure (ethanol/citrate buffer), produces fractionation of single apoptotic cells into multiple apoptotic bodies (not shown). The presence of nuclear fragments (DNA) in these apoptotic bodies allows the PI staining, which leads to an overestimation of the percentage of apoptotic cells. The higher level of spontaneous cell death detected by PI staining in the control samples (5.5%) compared with the

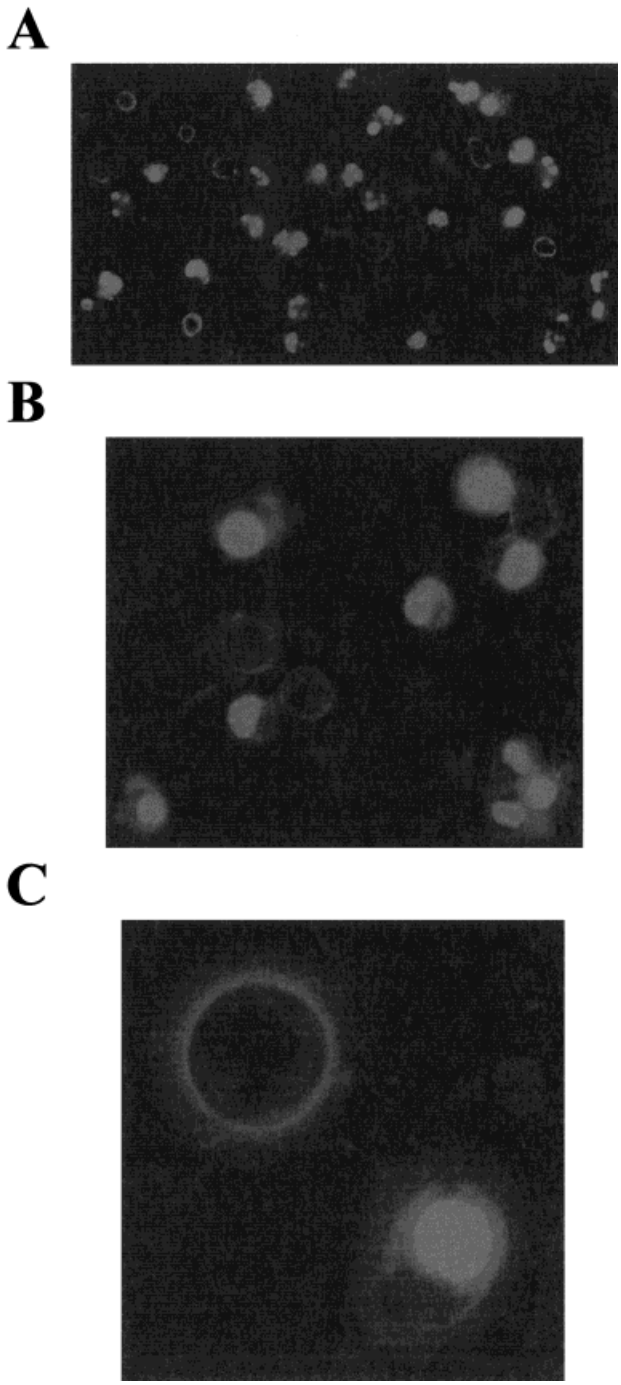

FIG. 4. PI staining in UV-treated cell lines was analyzed by fluorescence microscopy. Apoptotic cells are characterized by nuclear fragmentation and by strong staining of nuclei due to chromatin condensation. Living cells exclude PI and faint peripheral staining can be seen by simultaneous application of transmitted light phase-contrast and fluorescence microscopy. UV-treated **(A)** HL-60 cells (×500), **(B)** SK-N-MC neuroblastoma cell line (×660), and **(C)** Raji cells (×1,800) after 7 h of incubation.

supravital PI assay (2.0%; viable cells in culture are usually evaluated by trypan or PI exclusion) might also suggest that the overestimation of dead cells is intrinsic of the fixation/permeabilization procedure necessary for hypodiploid peak detection. It is not surprising that the results obtained with these two approaches do not strictly overlap because they measure different attributes of apoptotic cells and use thresholds to distinguish between apoptosis and debris that work in different ways.

The evaluation of apoptosis versus necrosis is an important parameter to distinguish between efficacy and toxicity of the treatment. Several chemotherapeutic drugs have been reported to induce apoptosis or necrosis depending on their concentrations. Therefore, correct evaluation of apoptosis versus necrosis becomes a fundamental parameter to adjust drug dose. Our technique, based on a progressive increase of the membrane permeability in apoptotic cells, allows the simultaneous detection of apoptosis and necrosis and has been used in many apoptotic models. This technique successfully examined classical camptothecin-induced cell death in HL-60 cells, apoptotic P815 cells induced by hyperthermia, apoptosis of Molt-4 cells in the absence of nucleosomal DNA fragmentation induced by staurosporin (15), tiazofurin-induced K562 apoptotic cells (35), apoptotic cell death of senescent megakaryocytes (3), and dexamethasone-induced apoptotic thymocytes (22). Other groups using different dyes such as 7-amino-actinomycin D (7-AAD), ethidium bromide (EB), and Hoeschst 33342 (HO342) found a similar progressive impairment of the plasma membrane barrier (24,36–41). Therefore, the data suggest that the increase of membrane permeability to different dyes is a hallmark of apoptotic cell death.

Annexin V, known to detect membrane alterations of apoptotic cells that are believed to appear earlier than the hypodiploid peak and light scatter changes, is widely used to detect apoptosis. The complete overlapping of Annexin V-positive cells with the PI^{dim} population demonstrates that the supravital PI staining technique allows the detection of apoptosis as early as Annexin V. However, although the Annexin V assay is widely used, it has several limitations. In fact, PS residues are exposed even in the absence of apoptosis, for example, in aging erythrocytes (42), in activated platelets (43–45), or after stimulation of leukemic cells (46), generating Annexin-positive cells that prevent their correct quantification of apoptotic cells by this technique. Moreover, in adherent cells, the evaluation of apoptosis by Annexin V is not reliable because trypsinization or scraping induces cell damage and high numbers of cells staining positive for Annexin V (23,26 and not shown). A fixation step after Annexin V binding has been proposed to overcome this problem (23,26), but this assay does not allow the direct discrimination between apoptotic and necrotic cells. In this assay, the administration of PI after the detachment of the adherent cells does not allow discrimination between the apoptotic cells damaged by detachment procedures and the real necrotic cells. The high DNA-binding constant and slow dissociation rate of PI prevent the leakage of PI out of positive cells. The fact that the PI incorporation by apoptotic and necrotic cells is not a quick reversible phenomenon has allowed the introduction in our assay of a washing step, which is necessary to detect additional specific

immunofluorescence staining. We found that this washing step decreased FL2 background and increased discrimination between viable and apoptotic cells (not shown), allowing an ideal combination of PI staining with FITC- or PE-conjugated mAb detection. However, although both high and low density antigens detected by FITC-conjugated mAbs can be easily examined in combination with PI, the same may not be true for low density antigens detected by PE-conjugated mAbs. Due to problems of compensation, PE^{dim} markers may be harder to resolve. Moreover, trypsin treatment after the PI staining does not interfere with the detection of apoptotic cells. It is likely that the membrane packing (47) and/or the transport function alterations (41) favor the passive diffusion of PI inside the cell when the dye in the medium is present at relatively high concentrations (at 50 μg/ml, but not at 5 μg/ml) and for long enough, as previously described (22). Inside the cell, the PI would bind nuclear structures, as detected by fluorescence microscopy, strongly enough to withstand the subsequent washing and trypsinization procedures. Nevertheless, the mechanical scraping procedure, which damages the plasma membrane, has to be avoided to impede leakage of PI out of the dead cells into the damaged ones.

In our experience, most of the cells collected from supernatants of adherent cells after apoptosis induction were PI^{dim}, i.e., apoptotic cells. The fact likely reflects the loss of cell surface antigens and, in particular, of adhesion molecules, typical of apoptosis (6) and indicates that apoptotic cells tend to spontaneously detach from the flask. In this regard, it becomes important to pool the cells in suspension with the cells harvested after the detachment procedures, in order to obtain a correct quantitative analysis of apoptosis.

This paper describes an assay for measuring apoptosis, independent of nucleosomal DNA fragmentation (15), that is extendible to adherent cells. Given the similar sensitivity, simplicity, and reliability, the supravital PI assay might be preferable to Annexin V staining. It is cheaper and, in contrast to Annexin V-FITC, it works with adherent cells. Finally, the supravital PI assay is associated with increased membrane permeability of apoptotic cells. It allows a quantitative analysis of apoptosis on unfixed cells, making it possible to evaluate cell surface markers or to study other features of apoptotic cells such as cytoplasmic functions after sorting.

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