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Detection of Localized Caspase Activity in Early Apoptotic Cells by Laser Scanning Cytometry

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Background: Caspase activation is a critical early step in the onset of apoptosis. Cell-permeable fluorogenic caspase substrates have proven valuable in detecting caspase activation by flow cytometry. Nevertheless, detection of early low-level caspase activation has been difficult using conventional area or peak fluorescence analysis by flow cytometry, despite the apparent presence of these cells as observed by microscopy. We describe a method utilizing maximum fluorescence pixel analysis by laser scanning cytometry (LSC) to detect early apoptotic cells.
Methods: The PhiPhiLux-G_1D_2 caspase 3/7 substrate was used in combination with DNA dye exclusion and annexin V binding to identify several stages of apoptosis in EL4 murine thymoma cells by both traditional flow and LSC. LSC analysis of maximum pixel brightness in individual cells demonstrated an intermediate caspase-low subpopulation not detectable by flow or LSC integral analysis. LSC analysis of caspase activity was then carried out using the larger UMR-106 rat osteosarcoma cell line to determine if this apparent early caspase activity could be correlated with localized, punctate caspase activity observed by microscopy.
Results: The caspase-low subpopulation found in apoptotic EL4 cells was also observable in UMR-106 cells. Relocation to cells with low fluorescence due to caspase activity and subsequent examination by microscopy demonstrated that these latter cells indeed show punctate, highly localized caspase activation foci that might represent an early stage in caspase activation.
Conclusions: Cells with low-level, localized caspase expression can be detected using maximum pixel analysis by LSC. This methodology allows an early step of apoptotic activation to be resolved for further analysis. Cytometry 47:81–88, 2002. © 2002 Wiley-Liss, Inc.

Key terms: apoptosis; caspase; LSC

The importance of apoptosis or programmed cell death in cell biology has mandated the development of accurate assays capable of measuring this phenomenon. Cell death assays based on flow cytometry have proven particularly useful; they are rapid, quantitative, and often can be carried out in heterogeneous populations in combination with fluorescence immunophenotyping (1). The multiparametric nature of flow cytometry also allows the detection of more than one cell death characteristic to be combined in a single assay. For example, apoptosis assays that utilize DNA dyes as plasma membrane permeability indicators (such as propidium iodide [PI]) can be combined with assays that assess different cellular responses associated with cell death, including mitochondrial membrane potential and annexin V binding to "flipped" phosphatidylserine (PS; 2–4). Combining measurements for cell death into a single assay has proven valuable in delineating apoptosis into multiple steps.

Recognition of the central role of caspases in the death process has led to the development of assays that can measure these important enzymes in situ. Caspase activation represents one of the earliest known markers for the onset of cell death (5). In most cases, caspase activation precedes cell permeability alterations and DNA damage whereas cytoskeletal collapse and PS flipping are often more concurrent. Loss of mitochondrial membrane generally occurs prior to caspase activation (6–11). Several fluorogenic assays have been developed for in situ analysis of caspase activation in intact cells (12–15). One recently described method is the use of the cell-permeable fluorogenic caspase 3/7 substrate PhiPhiLux (15). This reagent consists of an 18-amino acid peptide constituting the recognition and cleavage sequence from CPP32 (PARP)(REF), a physiological target for caspase 3 (16). The substrate is homodoubly labeled with fluorophores on opposite sides of the molecule such that its fluorescence is largely quenched (17). After the substrate enters a cell by passive diffusion and is cleaved by caspase 3 (or 7), the highly fluorescent fragments will be retained on the side of the

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membrane where the cleavage took place. This is due to the fact that these singly labeled peptides have a very low membrane permeability compared with the intact substrate. Although some diffusion will occur over an extended period of incubation, the presence of a fluorescence signal largely indicates the location of the caspase activities (Packard et al., unpublished data; 15). Detection of caspase activation with PhiPhiLux can also be combined with other assays for cell death, including annexin V binding and DNA dye exclusion. Several laboratories have used this reagent successfully to detect early apoptotic cells by fluorescence microscopy and flow cytometry (15).

In the immune system, the onset of apoptosis can be extremely rapid. Several authors have noted by flow cytometric analysis that cell death characteristics such as DNA degradation, morphology, and PS flipping result in the detection of few cells with an "intermediate" death phenotype. Cells are either nonapoptotic or possess the full complement of apoptotic characteristics, with two clearly definable populations and few intervening (1,2,4). As will be shown initially in this paper, this is also true of caspase activation as measured by flow cytometry. Nevertheless, we have observed microscopically what may be very early apoptotic cells following labeling for caspase activation; these cells show very low levels of nonuniform, punctate, and highly localized caspase activity (18). This is consistent with other studies that suggest nonuniform, organelle-specific caspase localization during the early stages of caspase cascade activation (19,20). The low overall fluorescence of these individual cells makes detection by traditional flow cytometry difficult. We describe the analysis of caspase activity by laser scanning cytometry (LSC), a derivative technology of traditional flow cytometry that allows a more sophisticated image-based analysis of individual cells (21). LSC has been used successfully in a variety of studies measuring both cell proliferation and apoptosis (22–25). This technique has allowed us to identify localized foci of caspase activation in early apoptotic cells, enabling these cells to be identified and subjected to further analysis.

MATERIALS AND METHODS

Cells and Cell Culture

EL4 murine thymoma cells were used for traditional flow cytometry and LSC analysis and UMR-106 rat osteosarcoma cells were used for LSC. The EL4 cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and it was passaged serially in RPMI 1640 with 10% fetal bovine serum (FBS). The UMR-106 cell line was also obtained from the ATCC and maintained in Dulbecco's minimum essential medium (DMEM) with 10% FBS. UMR-106 cells were passaged every 3 days by trypsinization. Apoptosis was induced in EL4 cells by treatment with actinomycin D at 2 μg/ml for 8 h. Apoptosis in UMR-106 cells was induced by treatment with valinomycin at 1 μM for 16 h. For most experiments, UMR-106 cells were plated onto four or eight-well glass tissue culture slides (Nunc, Naperville, IL) for subsequent analysis by LSC.

Cell Death Assays

Characterization of apoptosis in EL4 and UMR-106 cells was multiparametric, with labeling for DNA dye exclusion, annexin binding, and caspase activity. Following culture with or without drug treatment, EL4 cells were pelleted by centrifugation and the supernant decanted; for UMR-106 cells, culture medium was removed from the tissue culture slides upon which the cells were grown. Medium containing the fluorogenic caspase 3/7 substrate PhiPhiLux-G_1D_2 (DEVD ↑ GI consensus substrate peptide; OncoImmunin, Gaithersburg, MD) was added directly to cells in a volume of 50 μl. The cells were incubated at 37°C for 30 min followed by the addition of allophycocyanin (APC)-conjugated annexin V (Caltag, Burlingame, CA) directly to the cells in PhiPhiLux-containing medium. This was followed by a further incubation of 15 min at room temperature, one wash with Hank's balanced salt solution (HBSS) containing 2% FBS (hereafter referred to as wash buffer), and resuspension in the DNA binding dye 7-aminoactinomycin D (7-AAD) at 5 μg/ml. 7-AAD was substituted for the PI normally used to discriminate later apoptotic cells with PhiPhiLux due to the former's greater cell permeability and ability to exclude a larger fraction of advanced apoptotic cells. For traditional flow cytometric analysis, cells were analyzed within 30 min of 7-AAD addition. For EL4 cell analysis by LSC, cells were gently fixed in 0.5% paraformaldehyde following PhiPhiLux, annexin V, and 7-AAD labeling, centrifuged, decanted, and diluted 1:1 with mounting medium (Prolong AntiFade, Molecular Probes, Eugene, OR) containing 7-AAD at 5 μg/ml. The cells were then mounted on slides and analyzed within 1 h of mounting. For adherent UMR-106 cells, medium was removed from tissue culture slides following PhiPhiLux, annexin V, and 7-AAD labeling and the cells were fixed with 0.5% paraformaldehyde in phosphate-buffered saline (PBS). The fixative was then removed and a small volume of mounting medium with 7-AAD (5 μg/ml) was added, followed by coverslip mounting and analysis within 1 h.

Flow Cytometry

EL4 cells were analyzed on a Becton-Dickinson FACSCalibur benchtop flow cytometer (BD Biosciences, San Jose, CA) equipped with an air-cooled 15-mW 488-nm argon ion laser (for scatter determination, PhiPhiLux, 7-AAD, and PI excitation) and a 5-mW 635-nm red diode laser (for APC-annexin V excitation). PhiPhiLux-G_1D_2 was detected through a 530/30-nm fluorescein isothiocyanate (FITC) filter and 7-AAD and PI were detected through a 650 long pass filter. APC-annexin V was also detected through a 650 long pass filter. Data were acquired with CellQuest acquisition software, version 3.3 (BD Biosciences). All scatter measurements were made on a linear scale and all fluorescence measurements were made on a four-decade log scale. A total of 50,000 events were collected for each sample. Data were analyzed with WinMDI,

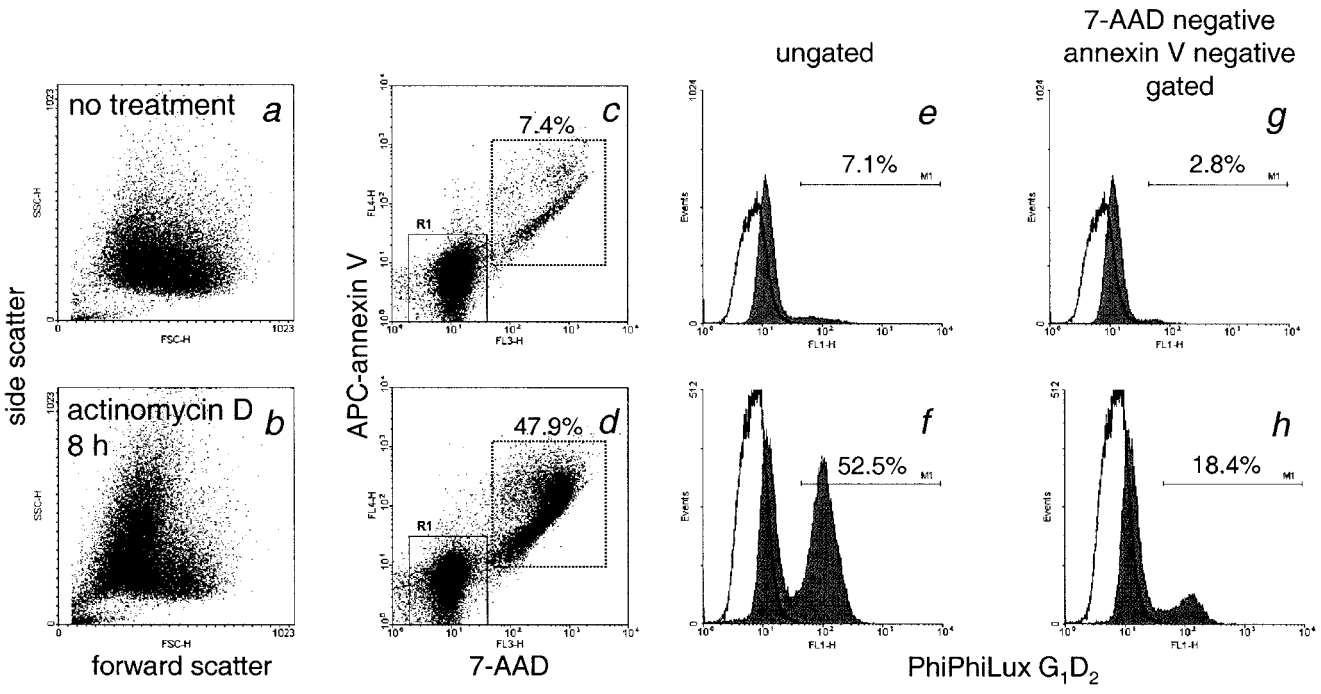

FIG. 1. Analysis of EL4 cell death by flow cytometry. EL4 cells were incubated with vehicle control (a,c,e,g) or with actinomycin D at 2 μg/ml for 8 h (b,d,f,h) followed by PhiPhiLux, APC-annexin V, and 7-AAD labeling as described in the Materials and Methods. **a,b:** Forward versus side scatter cytograms. **c,d:** 7-AAD fluorescence versus side scatter cytograms. **e,f:** PhiPhiLux fluorescence histograms for all cells. **g,h:** PhiPhiLux fluorescence histograms gated for 7-AAD-negative and APC-annexin V-negative cells (R1 in c,d). Dotted region shows the percentage of apoptotic events based on 7-AAD and annexin V fluorescence (c,d). Filled peaks indicate PhiPhiLux labeling and open peaks indicate unlabeled controls (e-h). Brackets show the percentage of apoptotic events based on PhiPhiLux fluorescence (e-h). All scatter analyses were made on linear scale and all fluorescence measurements were made on four-decade log scales. A total of 50,000 events were collected for each sample.

version 2.8 (Dr. Joseph Trotter, BD Pharmingen, San Diego, CA).

LSC

EL4 cells in suspension and adherent UMR-106 cells were analyzed on an LSC 2 laser scanning cytometer (Compucyte, Cambridge, MA) equipped with an air-cooled 15- mW 488-nm argon ion laser for PhiPhiLux and 7-AAD excitation and with a 25-mW 632-nm HeNe laser for APC-annexin V excitation. PhiPhiLux-G_1D_2 and 7-AAD were detected through a 530/30-nm FITC filter and through a 650 long pass filter, respectively. APC-annexin V was also detected through a 650 longpass filter. Data were acquired and analyzed with Wincyte acquisition software, version 3.4 (Compucyte). Scatter measurements were made on a linear scale and measurements of APC-annexin V, 7-AAD, and PhiPhiLux were made on a log scale. A total of 5,000 events were collected for each sample.

Photomicrography

Brightfield, conventional epifluorescence and LSC relocation-based epifluorescence photomicrography were carried out with the LSC-mounted Olympus BX50 epifluorescence microscope equipped with a Photometrix CoolMax CCD camera (Roper Scientific,Trenton, NJ). Image acquisition was carried out using IP-LAB image analysis software (Scanalytics, Fairfax, VA) using custom-written Windows-based programming language subroutines (BioVision, Exton, PA).

RESULTS

Caspase activation represents one of the earliest known steps in the cell death process (5). In this study, we have used the fluorogenic caspase 3/7 substrate, PhiPhiLux-G_1D_2, in conjunction with fluorochrome-conjugated annexin V and the DNA binding dye, 7-AAD, to measure cell death in the cell types described below. The resulting apoptotic subpopulations were then analyzed by flow or LSC.

Previous flow cytometry studies have shown with several cell types that use of PhiPhiLux enables clear distinction between nonapoptotic and apoptotic subpopulations with well-separated peaks and few intervening events (15). This is shown in Figure 1 for EL4 cells induced to undergo apoptosis by actinomycin D treatment. When analyzed for forward versus side scatter (Fig. 1a,b), a fraction of the EL4 cell culture showed a predictable reduction in forward scatter signal and an increase in side scatter upon apoptotic induction. Labeling for PS flipping from the plasma membrane inner to outer leaflet with APC-conjugated annexin V and cell permeability with 7-AAD (Fig. 1c,d) also showed different stages of apoptosis by both of these criteria. Caspase 3 activity as measured by PhiPhiLux labeling for all cells (no gating) is shown in

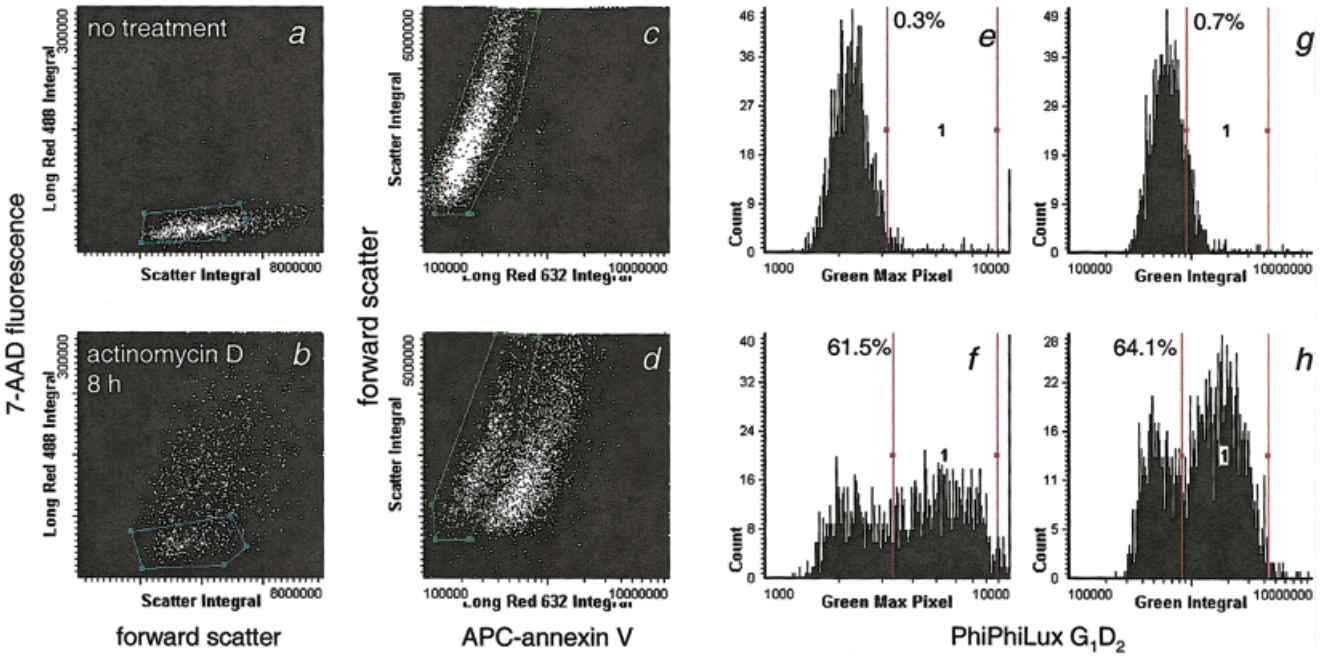

FIG. 2. Analysis of EL4 cell death by LSC. EL4 cells were incubated with vehicle control (a,c,e,g) or with actinomycin D at 2 μg/ml for 8 h (b,d,f,h) followed by PhiPhiLux, APC-annexin V, and 7-AAD labeling. **a,b:** Scatter integral (forward scatter) versus 7-AAD fluorescence cytograms. **c,d:** APC-annexin V fluorescence versus scatter integral cytograms. **e,f:** PhiPhiLux max pixel fluorescence histograms for cells gated for 7-AAD-negative and APC-annexin V-negative cells (gates shown on a-d). **g,h:** PhiPhiLux integral fluorescence histograms for cells gated for 7-AAD-negative and APC-annexin V-negative cells. Scatter and 7-AAD measurements were made on linear scale, whereas APC-annexin V and PhiPhiLux measurements were made on log scale. A total of 5,000–10,000 events were collected for each sample.

Figure 1e, f. When cells were gated for negative labeling by annexin V and 7-AAD, a significant portion of this population still showed caspase 3 activity (Fig. 1g,h), with almost 20% of the cells being caspase positive following actinomycin D treatment. Although PI is recommended often for use with PhiPhiLux to define later apoptotic cells and to quantify more accurately the apoptotic cell subpopulation, 7-AAD was substituted in these experiments to define more clearly the earlier apoptotic cell population. PI is less cell permeable and would give a higher number of caspase-positive cells (due to the presence of 7-AAD-positive PI-negative cells possessing caspase activity) that were excluded intentionally from this analysis (15). This experiment illustrated two important points. First, caspase activation is a very early step in cell death, preceding early plasma membrane permeability and PS flipping. Second, the difference in caspase activity between apoptotic and nonapoptotic cells is significant and easily distinguishable, with few intervening events detectable by traditional flow cytometry. This bimodality has been found previously to be true of many apoptotic phenomena; the onset of cell death and the conversion of cells from a nonapoptotic to apoptotic phenotype usually occurs with few detectable intermediate states (1,2,4).

Nevertheless, previous analyses of caspase-active cells by confocal microscopy has suggested that intermediate stages of caspase activation may be observable (18). Previous confocal microscopy of PhiPhiLux-positive cells has demonstrated early apoptotic cells with nonuniform, punctate caspase activity apparently localized to the cytoplasm and likely compartmentalized in vesicles (18). Traditional peak or area analysis by flow cytometry was not sufficiently sensitive to detect this early caspase activation. This was likely due to both its insignificant portion of total cellular fluorescence under a total area measurement and its rapid time-of flight through the laser resulting in a low peak fluorescence sensitivity. Because traditional flow cytometry was not successful at detecting these early-stage apoptotic cells, EL4 cells were labeled as described above and analyzed in fixed suspension by LSC. In addition to analyzing the integrated fluorescence of a cell, the LSC can collect fluorescence data for individual pixels within a cell area and express this as the fluorescence signal (termed maximum pixel or max pixel analysis). Because cells with intermediate levels of caspase activity exist, max pixel analysis was anticipated to be more sensitive for their detection than traditional flow cytometric peak or area analysis (21,26).

Cells were scanned for scatter integral (analogous to forward scatter) versus 7-AAD fluorescence (Fig. 2a,b). The later apoptotic cells show a predictable increase in 7-AAD fluorescence. When analyzed for scatter integral versus APC-annexin V binding (Fig. 2c,d), the apoptotic cells were also readily identifiable. As for the EL4 cells in Figure 1, the 7-AAD and annexin V-negative cells were then gated and their caspase 3 activity measured (Fig. 2e-h). Two acquisition modes were used. Green max pixel analysis (Fig. 2e,f) expresses fluorescence signal data as

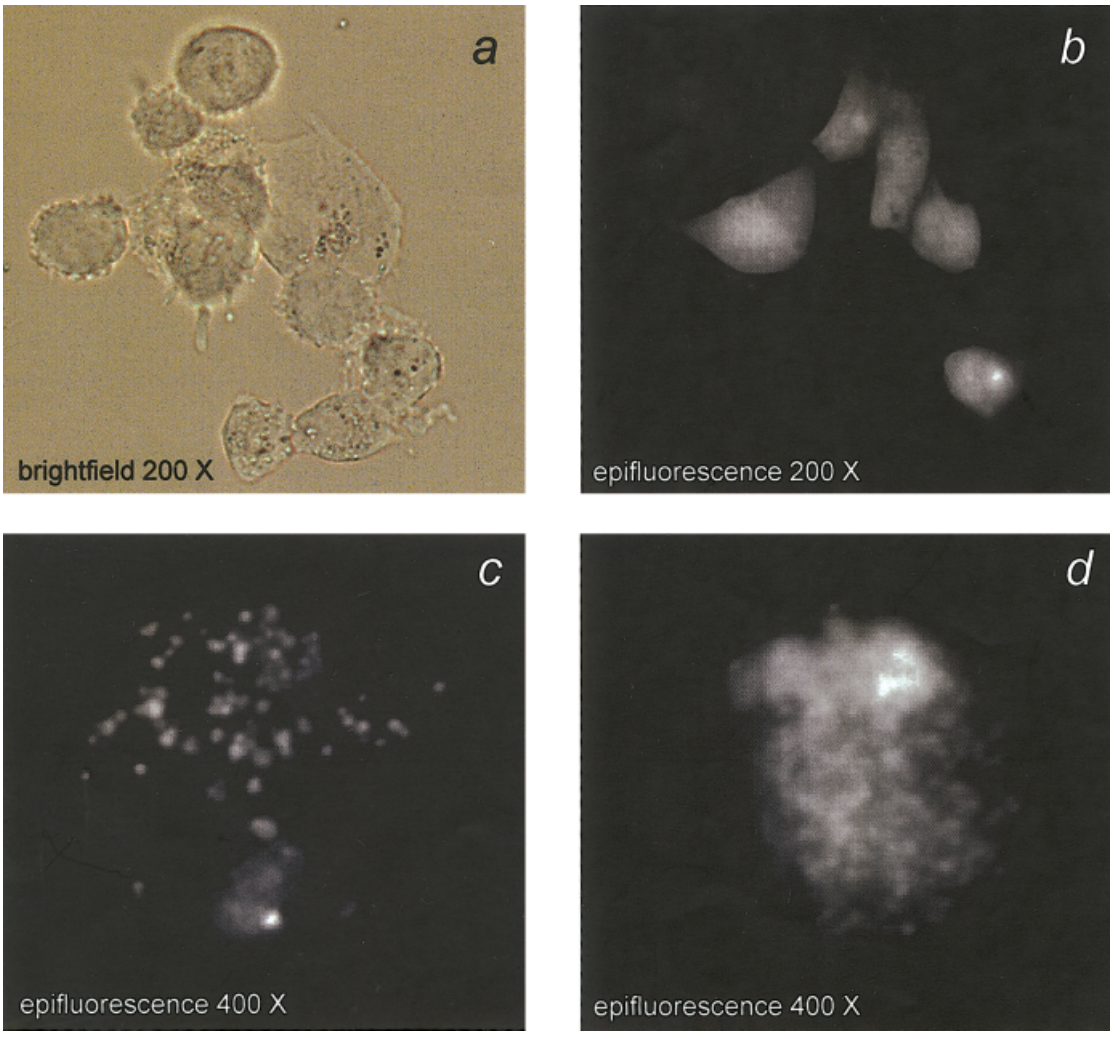

FIG. 3. Photomicrographs of UMR-106 rat osteosarcoma cells. **a:** Brightfield photograph (200×) of viable UMR-106 cells. **b:** Epifluorescence photograph (200×) of viable and apoptotic UMR-106 cells following treatment with valinomycin at 1 μM for 16 h and labeling with PhiPhiLux G_1D_2. **c:** Epifluorescence photograph (400×) of UMR-106 cells with a low level of localized caspase activity. **d:** Epifluorescence photograph (400×) of UMR-106 cells with an intermediate level of localized caspase activity. All photographs were acquired with the LSC-coupled Olympus BX50 epifluorescence microscope equipped with a Roper Scientific/Photometrix cooled CCD camera controlled by Scanalytics IPLAB image acquisition/analysis software.

the brightest pixel within the scan area containing a cell. Cells with caspase activity in very localized regions would be expected to be distinguished more easily by max pixel analysis. Green integral analysis (Fig. 2g,h) expresses fluorescence signal data as the integral of all pixels within a cell scan area. Both analysis modes showed distinct nonapoptotic and apoptotic subpopulations. Nevertheless, green max pixel analysis gave better separation between the cell types with a broader "valley" between the peaks, suggesting that very early apoptotic cells might fall within this region. Moreover, cells in this area might possess the punctate caspase activity previously seen by confocal microscopy.

Unfortunately, EL4 cells are not large, making subcellular caspase activity localization with LSC analysis difficult. The much larger and adherent UMR-106 rat osteosarcoma cell line was analyzed in this manner. Figure 3a shows UMR-106 cells with brightfield illumination. These cells readily undergo caspase-associated apoptosis when treated with the ionophore valinomycin for 16 h. Figure 3b shows valinomycin-treated cells that were subsequently examined for caspase 3 activity with PhiPhiLux. Caspase activity was relatively uniform throughout the cytoplasm of apoptotic cells and was virtually absent in uninduced cells (barely visible in Fig. 3b). Caspase activity in UMR-106 cells is also an early event; it precedes cell permeability and morphological changes that ultimately result in cell rounding and release from the culture surface. Hence, numerous caspase-positive cells still adhered to the culture slide, making their detection possible.

In a fraction of treated UMR-106 cells, however, nonuniform patterns of caspase activity could be observed. Figure 3c,d shows two stages of nonuniform punctate caspase activity. Fluorescence patterns in these cells are

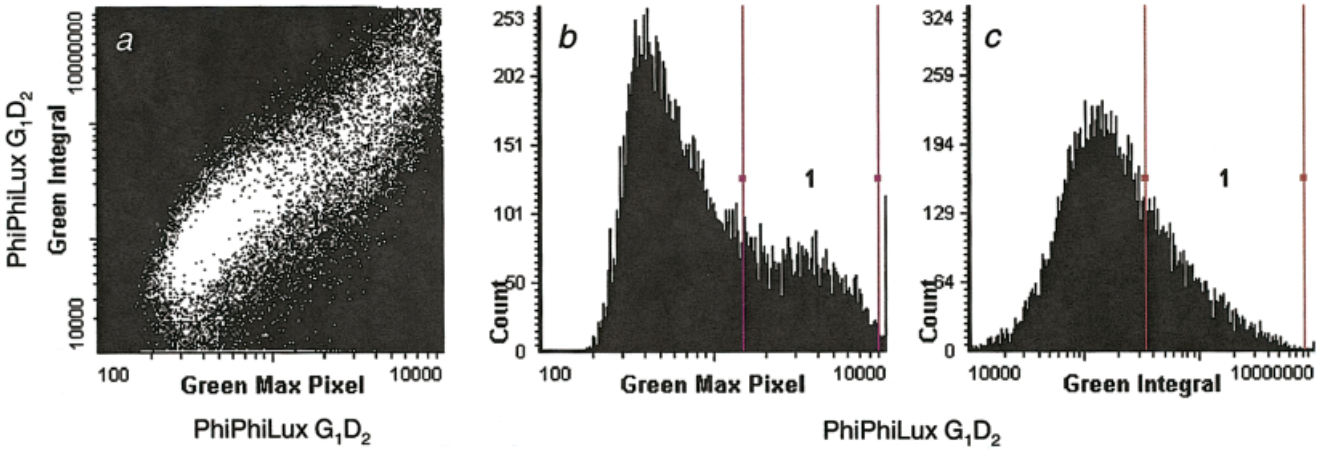

FIG. 4. Analysis of UMR-106 cell death by LSC. UMR-106 cells were incubated with valinomycin at 1 μM for 16 h followed by PhiPhiLux, APC-annexin V, and 7-AAD labeling as described in the Materials and Methods. **a:** PhiPhiLux max pixel versus integral cytogram. **b:** PhiPhiLux max pixel fluorescence histograms for cells gated for 7-AAD-negative and APC-annexin V-negative cells. **c:** PhiPhiLux integral fluorescence histograms for cells gated for 7-AAD-negative and APC-annexin V-negative cells. Regions on histograms indicate caspase-positive cells based on controls (not shown). A total of 5,000–10,000 events were collected for each sample.

consistent with the early stages of caspase activation. In the cell shown in Figure 3c, the bright but low number of caspase activity foci might not make this cell distinguishable from nonapoptotic cells when analyzed by peak or area fluoresence intensity by conventional flow cytometry.

To determine if this was the case, treated UMR-106 cells were analyzed by LSC using the detection scheme described above (Fig. 4). Unlike EL4 cells, virtually no scatter-apoptotic 7-AAD or APC-annexin V-positive cells were present on the slides, because they had likely rounded up and were removed during the washing steps. The cytogram for PhiPhiLux green integral versus max pixel is shown in Figure 4a, with histograms for both characteristics in Figure 4b,c. Although caspase activity was detectable by both modes of analysis, the peak separation and intervening valley were much greater for max pixel analysis; integral analysis gave a shoulder that was not separated clearly from the nonapoptotic subpopulation.

The greater separation between caspase-negative and caspase-positive cells using max pixel analysis permitted subsequent relocation analysis of these intermediate cells. A unique feature of LSC is the recording of each cell's X-Y coordinate location as part of the FCS data file structure. Cells identified by their scatter and/or fluorescent characteristics can therefore be "relocated" and automatically repositioned under the microscope for morphological analysis. Relocation analysis for apoptotic UMR-106 cells in shown in Figure 5. A gating region was placed in the valley between the nonapoptotic and apoptotic subpopulation, and a series of these cells were relocated and photographed (Fig. 5a). The cells thus identified showed very distinctive nonuniform, punctate caspase activity, some at very low levels. Figure 5b shows the relocation of the apoptotic cells with the highest level of caspase activity. The cells show more uniform caspase activity. This analysis shows that max pixel analysis of caspase activity, particularly in large cells such as UMR-106 cells, allowed the identification of cells with extremely low levels of caspase activity, likely a precursor to full caspase activation.

DISCUSSION

The capability to detect apoptosis at its earliest stages is critical to understanding this important physiological process. Most of the original assays for apoptotic death (including analysis of increased membrane permeability, cytoskeletal collapse, and DNA damage) represented terminal events in advanced cell death (1,2,4,6). Considerable deterioration of cellular physiology has occurred already at this point, making meaningful analysis of the cellular responses to the apoptotic stimuli in these cells less significant. Clearly, monitoring and characterizing cells undergoing apoptosis with early apoptosis markers would be invaluable in delineating the molecular events and pathways involved in programmed cell death. We and others have measured the temporal onset of cell death using the PhiPhiLux fluorogenic caspase 3 substrate simultaneously with other early indicators of cell death, including annexin V binding to flipped PS and incorporation of DNA binding dyes (15). In the EL4 and UMR-106 cell systems described above, caspase 3/7 activation occurred earlier than either PS flipping or loss of membrane integrity. Caspase activation therefore represented an apoptotic event that was distinct from other early indicators of cell death.

Because activation of the caspase cascade is one of the central cellular events of apoptosis, this temporal correlation between downstream caspase activation and PS flipping prompted us to seek a more sensitive means to detect the onset of caspase activation using the same cell-permeable fluorogenic caspase substrate. Earlier con-

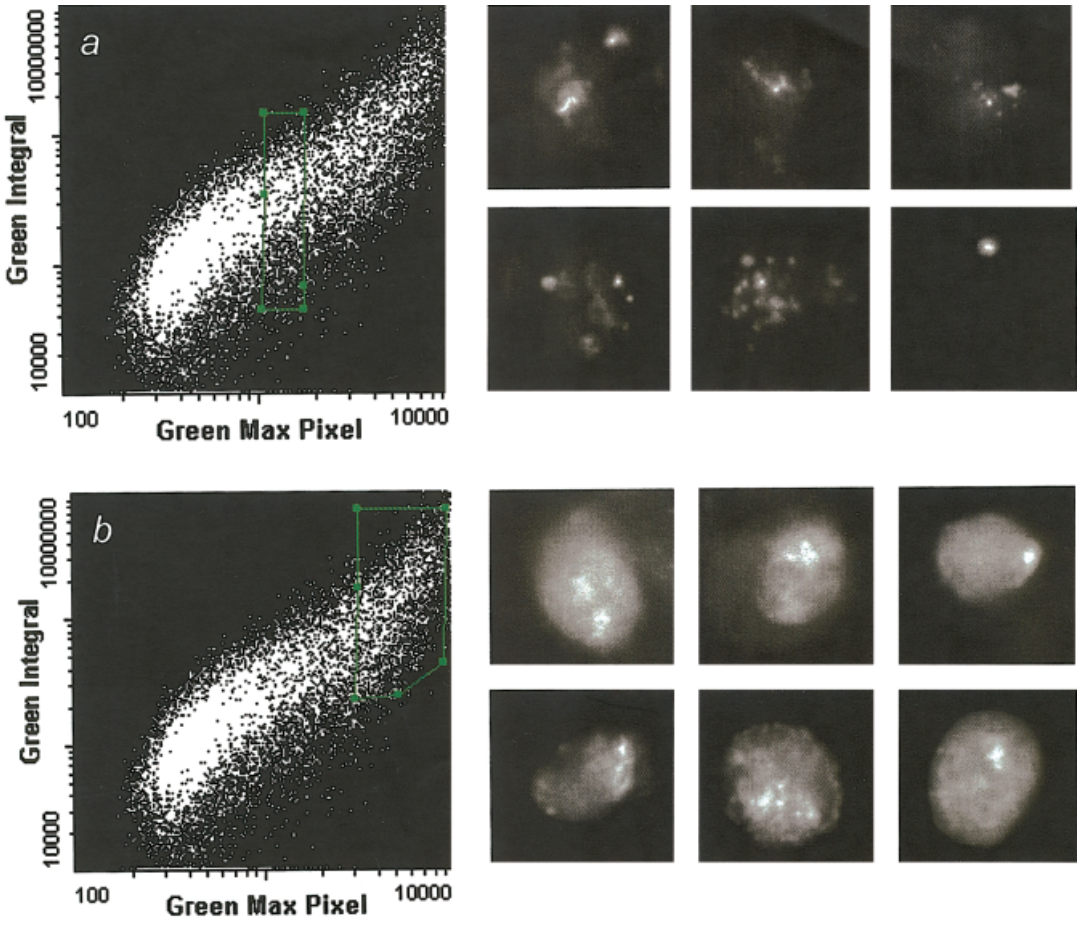

FIG. 5. Relocation analysis of UMR-106 cells expressing low levels of caspase activity. UMR-106 cells were incubated with valinomycin at 1 μM for 16 h followed by PhiPhiLux, APC-annexin V, and 7-AAD labeling as described in Figure 4. Gates were then drawn to enclose either caspase-low cells (**a**) or caspase-high cells (**b**) based on PhiPhiLux max pixel analysis and relocation analysis carried out. Six representative images are shown to the right of each cytogram.

focal microscopy observation of cells undergoing apoptosis characterized by the same downstream caspase substrate indicated that the downstream caspase 3/7 activities are largely cytoplasmic but not necessarily uniform throughout the cytoplasm, suggesting that early caspase activation might be represented by nonuniform, punctate foci of caspase activity. This is consistent with other studies suggesting nonuniform, organelle-specific localization of caspases during cell death (18–20). PhiPhiLux-G_1D_2 has the ability to cross not only the plasma membrane but also all intracellular membranes, giving it entry to all cytoplasmic vesicles. However, once the substrate is cleaved by the target caspase, the digested substrate shows reduced membrane permeability and is retained largely on the side of the membrane where the cleavage took place. Therefore, PhiPhiLux fluorescence quite accurately reflects caspase localization. The ability of PhiPhiLux to show caspase localization led us to hypothesize that nonuniform caspase activity in cells may represent an early stage of apoptosis-associated caspase activation.

Analysis of apoptosis in both EL4 and UMR-106 cells by conventional flow cytometry demonstrated populations of caspase-positive cells that were delineated clearly from nonapoptotic cells. Nevertheless, flow cytometry showed few intermediate events that might represent cells with nonuniform, punctate caspase activity, probably due to the fraction of caspase fluorescence in the total area integral of the analyzed cell, the short period in which the cell occupies the laser beam for sensitive peak analysis. Peak or area fluorescence measurements used in conventional flow cytometry were not well suited to detecting bright but highly localized fluorescence in individual cells. The unique imaging capabilities of the LSC permitted analysis of single bright pixels within a cell and expression of these signals as data entities (max pixel analysis; 21,22,26). We were able to use LSC max pixel analysis to identify cells with intermediate levels of caspase activation. When cells with this level of caspase activity were relocated and examined by epifluorescence microscopy, they showed punctate caspase activity that likely represented an early stage of caspase activation. They were visually distinct from cells with higher levels of PhiPhiLux activity, which showed more uniform activated caspase distribution.

The punctate appearance of this downstream caspase activity suggests that the active downstream caspases were localized within subcellular compartments and/or vesicles. One possible explanation for this localization may be the onset of zeiotic reorganization, an early apoptotic process that may be associated with alterations in cell volume and is characterized by significant cytoplasmic perturbation (27). Punctate caspase 3/7 activity may reflect and be related to the onset of zeiosis in early apoptotic cells. Zeiosis is a hallmark of apoptosis as defined by morphological analysis. Nevertheless, it has received little attention from many apoptotic studies due to the lack of a good quantitative method for detection (18).

Multiparametric analysis of cells undergoing apoptosis has allowed identification of various temporal stages of apoptosis at the single cell level when parameters derived from the same single cells are compared. We have shown that max pixel analysis carried out by the LSC technique provides a simple method to quantify localized caspase activity in apoptotic cells. This localized caspase activity may also represent the onset of zeiosis, a significant but poorly defined apoptotic process. The present approach should become a valuable tool for the study of both apoptosis and other cellular processes that demonstrate subcellular localization/distribution of proteases or enzymes.

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