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In Vitro Detection of Apoptotic Stimuli by Use of the HL-60 Myeloid Leukemic Cell Line

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The human histiocytic lymphoma line HL-60 has served as a model of myeloid cell differentiation and can be induced to differentiate along the neutrophil or monocytic lineage, depending on the external stimulus. The nondifferentiated cell line retains a premyeloid leukemic phenotype and is capable of anchorage-independent growth and proliferation. The role of apoptosis in the regulation of immunologic and inflammatory events associated with homeostasis and disease has been most intensively studied in lymphocytes. In the present study, nondifferentiated HL-60 has served as a model for studying myeloid cell apoptosis by investigating apoptotic changes induced by camptothecin, a DNA topoisomerase inhibitor, as well as physiologic stimuli, including ceramide analogs and a monoclonal antibody against the Fas antigen. Multiparameter flow cytometry was used to evaluate apoptosis by measuring changes in both side scatter and propidium iodide staining. The appearance of apoptotic cells was confirmed biochemically by measuring DNA endonuclease activity by both enzyme-linked immunosorbent assay quantitation and DNA ladder formation on agarose gels and morphologically with the detection of micronuclei by confocal laser microscopy. These studies demonstrate that HL-60 can serve as an in vitro model for the detection of physiologic and pharmacologic apoptotic stimuli and for understanding the early and late cellular changes associated with induction of the apoptotic program.

Apoptosis is a form of programmed cell death that plays a major role in both development and homeostasis and as a result of aberrant regulation can contribute to a variety of pathologic states. Assessment of apoptotic events at the cellular level includes both morphologic and biochemical changes, such as chromatin condensation, reduced cell volume, dilation of the endoplasmic reticulum, and nuclear fragmentation. Biochemical changes can include internucleosomal DNA cleavage, generating a characteristic ladder formation on gels, as well as significant changes in calcium influx (30, 32, 34, 35). The role of apoptosis in immunology and inflammation has been investigated, and its involvement in T-cell development, tolerance, and thymic selection continues to be of intense interest (18, 32). Glucocorticoid-induced apoptosis in T cells has served as a model in which the biochemical changes associated with apoptosis were investigated with a synchronized cell population (18, 26). Furthermore, the consequences associated with a defect in T-cell apoptosis have been demonstrated for the *lpr* and *gld* mutations in mice (3, 8, 31, 33). These mutations are associated with the appearance of double-negative T cells in lymph nodes, lymphoproliferation, and profound autoimmunity as evidenced by high titers of autoantibodies against both nuclear and matrix components (1, 27).

Apoptosis and B-cell development have also been investigated, and the role of the *bcl-2* gene in suppression of apoptosis was first recognized by chromosomal translocations associated with B-cell neoplasias mapped to the *bcl-2* locus (2, 17). Apoptotic changes are also apparent in myeloid cells and were first described for senescent neutrophils (29). In contrast to necrotic neutrophils, often observed at inflammatory lesions, apoptotic neutrophils retain cellular integrity, do not undergo lysosomal degranulation, and are phagocytized by macrophages through vitronectin and thrombospondin receptors (28). Apoptosis in myeloid cells can be observed in vitro following removal of necessary growth factors, including the colony-stimulating factors and gamma interferon, and may represent a defense mechanism of the macrophage against pathogenic organisms associated with intracellular parasitic infection (21, 22, 40).

While the cellular and molecular changes associated with apoptosis have been investigated in myeloid cells, a greater understanding as to which pharmacologic and physiologic stimuli result in induction of the apoptotic program is necessary. Thymocytes and T-cell lines have served as useful models for understanding the glucocorticoid-mediated induction of lymphocyte apoptosis. Myeloid cell apoptosis, in contrast, is less well understood, since primary cultures of neutrophils and monocytes are subject to both apoptotic and necrotic changes with culture alone. In the present study, the HL-60 myeloid leukemic cell line has been used in an attempt to further characterize both pharmacologic and physiologic agents which promote apoptosis. Apoptosis was defined by multiparameter flow cytometry, DNA ladder formation, confocal laser microscopy, and enzyme-linked immunosorbent assay (ELISA) quantitation of soluble DNA fragments by use of physiologic stimuli, including ceramide metabolites and activation of the Fas antigen, and a pharmacologic stimulus, the DNA topoisomerase inhibitor camptothecin. The results of these studies demonstrate that HL-60 offers a well-characterized in vitro system in which the effects of both pharmacologic and physiologic stimuli on the induction of myeloid cell apoptosis can be investigated.

MATERIALS AND METHODS

Cell system. HL-60 cells were maintained in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum, glutamine, and antibiotics. At the start of the experiments, cells in logarithmic phase were plated at 3×10^5/ml in RPMI 1640 medium with 0.5% fetal calf serum and incubated with 1 to 5 μM C_2-ceramide (*N*-acetylsphingosine), C_6-ceramide (*N*-hexanoylsphingosine), or C_8-ceramide (*N*-octanoylsphingosine) which had been dissolved in ethanol as 1 mM stocks (BioMol, Plymouth, Mass.). In other experiments, cells were incubated with 0.15 μM camptothecin (Sigma Chemical, St. Louis,

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Mo.) prepared as a 1.5 mM stock in dimethyl sulfoxide or with 1 to 50 ng of human anti-Fas (Upstate Biotech Industries, Lake Placid, N.Y.) per ml. Cells were incubated with the appropriate agents for 0 to 48 h and evaluated for apoptosis.

Flow cytometry. Cells were washed and processed for flow cytometry using propidium iodide staining based on minor modifications of established procedures (23, 26). Briefly, cells were centrifuged, resuspended in 1 ml of a 100-μg/ml propidium iodide (PI) solution in 0.1% sodium citrate and 0.1% Triton X-100, and incubated for 30 min on ice. Red fluorescent PI staining was evaluated with a FACSort flow cytometer (Becton Dickinson, Mountain View, Calif.) using a four-decade logarithmic scale for fluorescence detection. The peak fluorescent intensity was calibrated to channel 2000 in the logarithmic mode. Side, 90° scatter and fluorescent intensity in the FL-3 channel were used for multiparameter analysis. Ten thousand cells from each sample were analyzed, and both histogram and dual-parameter dot plot analyses were performed with LYSYS II software (Becton Dickinson).

Laser confocal microscopy. PI-stained hypotonic lysed cells were fixed on poly-L-lysine-treated slides and evaluated by laser confocal microscopy using an MRC-1000 UV confocal laser scanning image system (Bio-Rad, Richmond, Calif.) equipped with an argon-krypton laser and Gateway 2000 computer support. Representative images were captured and processed.

DNA analysis. Genomic DNAs were isolated from both control and camptothecin-treated cultures by standard procedures. Briefly, cells were removed from wells and centrifuged to remove culture medium. Cell pellets were resuspended in 100 μl of lysis buffer (1% sarcosine in 0.5 M EDTA containing 250 μg of RNase A per ml) and incubated for 30 min at 37°C. Proteinase K (final concentration, 100 μg/ml) was subsequently added, and incubation was continued overnight at 45°C. DNA fragmentation was detected by electrophoresis of 3×10^5 cell equivalents of each sample in 1.5% agarose gels. All electrophoretic analyses incorporated a 1-kb DNA ladder (Gibco BRL) for size determination. DNA bands were detected following ethidium bromide staining. Soluble DNA fragments were quantitated with an ELISA kit (Boehringer Mannheim, Indianapolis, Ind.) according to procedure described by the supplier.

RESULTS

When stained with PI under hypotonic conditions, HL-60 cells exhibit nuclear staining indicative of a diploid to tetraploid nuclear distribution (Fig. 1). Dual-parameter analysis using 90-degree side scatter in the linear mode and PI staining in the logarithmic mode demonstrated that between 85 and 95% of the cells could arbitrarily be placed in the lower right-hand quadrant, suggesting uniform fluorescence with a low level of side scatter. In contrast, when HL-60 cells were coincubated with 5 μM C_2-ceramide, 0.15 μM camptothecin, or 10 ng of anti-Fas per ml for 24 (camptothecin) or 48 (anti-Fas and C_2-ceramide) h, there was a significant shift in fluorescence to a hypodiploid level. There was a variable increase in the amount of side scatter, particularly with C_2-ceramide. The reduction in nuclear staining was associated with cells that morphologically displayed evidence of nuclear condensation and micronucleation. These changes occurred in the majority of the cells evaluated by both single- and dual-parameter flow cytometry.

The induction of apoptosis by the cell-permeable C_2-ceramide analog was dose dependent (Fig. 2). At the lowest concentration of ceramide evaluated (1 μM), there was a slight increase as determined by both histogram and dot plot analysis in cells characterized by a hypodiploid PI staining profile with an increase in side scatter (Fig. 2B) in comparison with the control (Fig. 2A). Higher concentrations of C_2-ceramide (2.5 and 5 μM) resulted in further increases in the number of cells characterized by an apoptotic phenotype (Fig. 2C and D). The percentage of HL-60 cells in the lower right-hand quadrant, representing nonapoptotic cells for each of the C_2-ceramide concentrations, was determined by using the appropriate statistical analysis within the LYSYS II software and compared with the results for a 5 μM concentration of two less-active cell-permeable ceramide analogs, C_6- and C_8-ceramide (Fig. 2E). Neither the C_6- nor the C_8-ceramide resulted in any significant reduction in the percentage of cells in the lower right quadrant, suggesting a structure-activity relationship among these analogs.

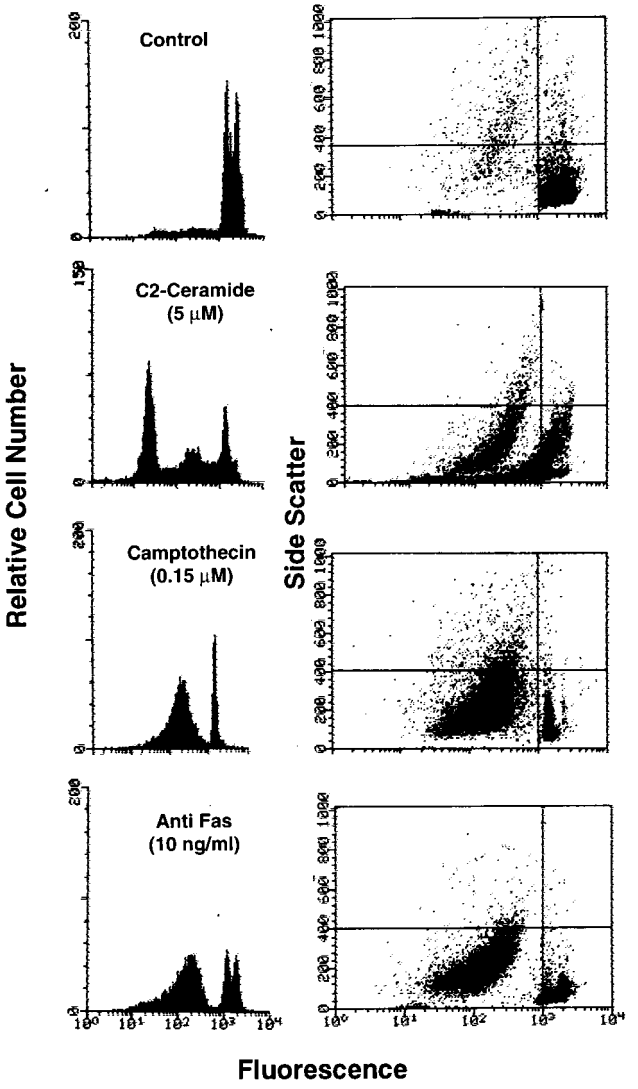

FIG. 1. Flow cytometric analysis of HL-60 apoptotic changes. HL-60 cells were incubated with medium alone (control) or with the indicated concentration of C_2-ceramide, camptothecin, or anti-Fas for 24 (camptothecin) or 48 (ceramide and anti-Fas) h. Cells were stained with PI, and 10,000 cells were evaluated by single-parameter flow cytometry using PI fluorescence (histogram) and dual-parameter flow cytometry with PI and 90° side scatter. Fluorescence intensities are indicated in the logarithmic mode (*x* axes). Apoptotic changes indicated by decreased fluorescence staining in comparison with the control were observed. The percentages of cells in the lower right quadrants for the various cell treatments were 88.9, 19.9, 21.2, and 28.2% for the control, C_2-ceramide, camptothecin, and anti-Fas, respectively.

Anti-Fas monoclonal antibody also triggered HL-60 apoptosis in a dose-dependent manner (Fig. 3). Minimal effects in comparison with the control were apparent with 1 ng of anti-Fas per ml. In contrast, anti-Fas at 10 and 50 ng/ml resulted in a more significant shift in PI staining. Interestingly, the anti-Fas-mediated changes in side scatter were less significant than the increases in side scatter observed with ceramide.

While the flow cytometric profiles of HL-60 cells treated with C_2-ceramide, anti-Fas, or camptothecin were consistent with apoptosis, it was necessary to evaluate these changes by other criteria. Accordingly, control and camptothecin-treated

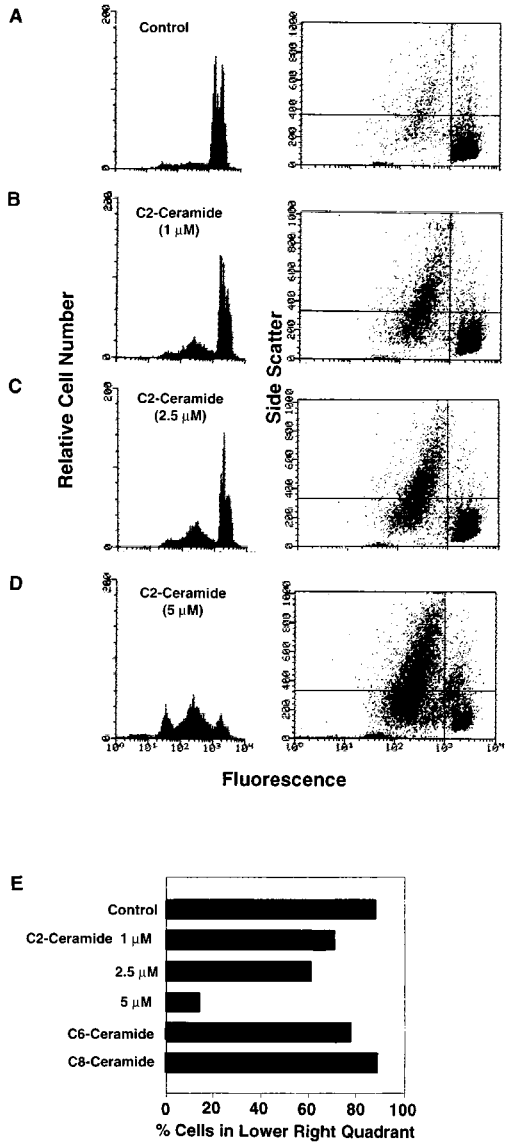

FIG. 2. Dose-dependent effects of C_2-ceramide on HL-60 apoptosis. HL-60 cells were incubated with medium alone (A) or the indicated concentrations of C_2-ceramide (B to D) for 48 h and processed for single- and dual-parameter flow cytometry as described in the legend to Fig. 1. The percentages of cells in each of the four quadrants were determined with the LYSYS II software package. The change in the percentages of cells in the lower right quadrant, reflecting nonapoptotic cells, is expressed graphically (E) for the above C_2-ceramide concentrations as well as for a 5 μM concentration of the less active C_6- and C_8-ceramide analogs. In five separate experiments with 5 μM C_2-ceramide, the variability in the percentages of cells in the lower right quadrant (nonapoptotic) varied between 14 and 29%.

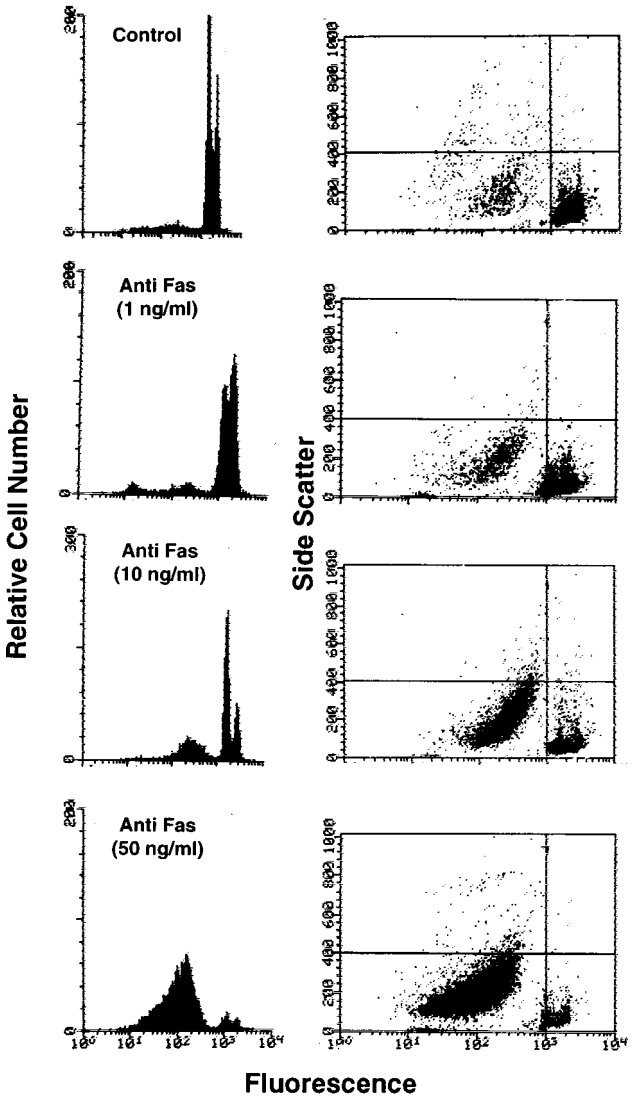

FIG. 3. Dose-dependent effects of anti-Fas antibody on HL-60 apoptosis. HL-60 cells were incubated with the indicated concentrations of anti-Fas antibody for 48 h and processed for both single- and dual-parameter flow cytometry as described in the legend to Fig. 1. The percentages of cells in the lower right quadrants for the various cell treatments were 91, 81.7, 28.2, and 5% for the control and 1, 10, and 50 ng of anti-Fas per ml, respectively.

HL-60 cells were examined by laser confocal microscopy for the detection of micronuclei and other morphologic changes in PI-stained nuclei. Nuclei in camptothecin-treated HL-60 cells exhibited micronucleation and smaller diameters than those of untreated controls (Fig. 4A and B). The appearance of micronuclei by laser confocal microscopy was consistent with the reduction in PI fluorescence demonstrated by flow cytometry.

Additionally, apoptosis is often associated with endonuclease activation resulting in internucleosomal breaks. The generation of these smaller DNA fragments was apparent from the characteristic ladder formation on agarose gels for camptothecin-treated HL-60 cells (Fig. 4C, lane 1) while the control HL-60 cells (Fig. 4C, lane 2) failed to exhibit a similar nucleosomal oligomer pattern. Finally, ELISA quantitation of soluble DNA fragments (Fig. 4D) revealed a significant increase in lysates from the camptothecin- and anti-Fas-treated cells compared with the parallel lysate controls. Therefore, HL-60 responds to both pharmacologic and physiologic stimuli associated with the activation of the apoptotic program as measured by flow cytometric, morphologic, agarose gel, and ELISA methods. HL-60 cells can thus serve as an in vitro system for the detection of novel apoptotic signals which may result in myeloid cell apoptosis.

DISCUSSION

Apoptosis as a fundamental biologic process involved in both homeostasis and the pathogenesis of many chronic and

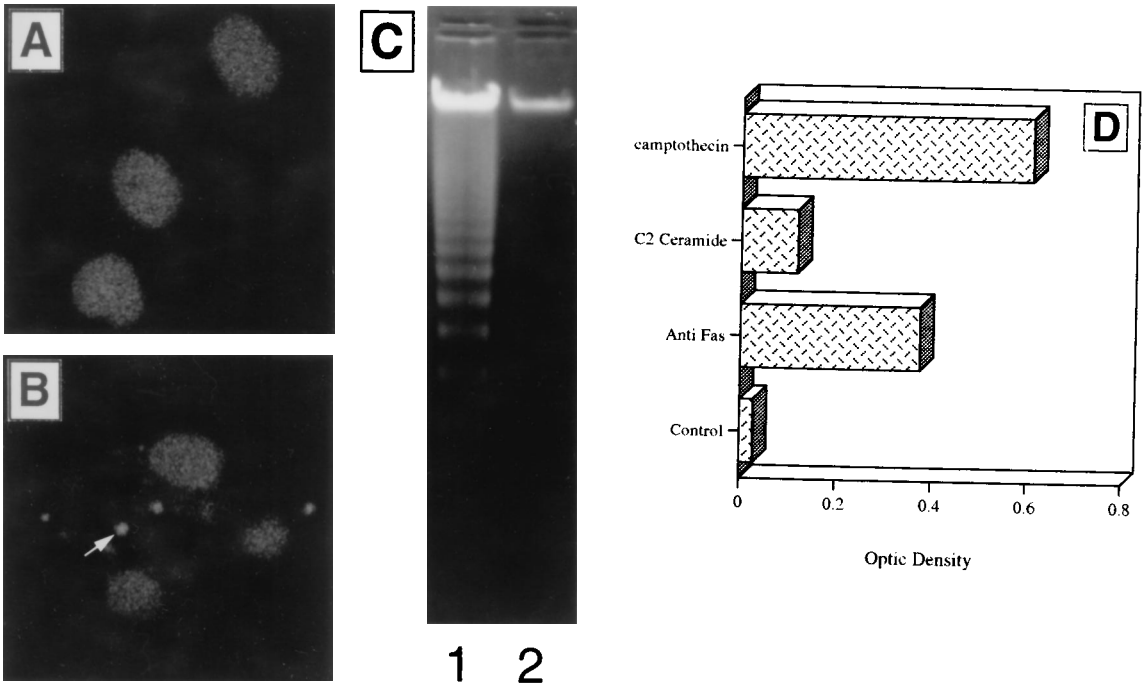

FIG. 4. Evaluation of HL-60 apoptosis by non-flow cytometric techniques. HL-60 was incubated with 0.15 μM camptothecin, 10 ng of anti-Fas per ml, or 2.5 μM C_2-ceramide for various intervals and processed for laser confocal microscopy (A and B), agarose gel analysis (C), and quantitation of soluble nucleosomal fragments (D). Laser confocal microscopy was performed on control (A) and camptothecin-treated (B) HL-60 cells after 24 h. DNA ladder formation for control and camptothecin-treated HL-60 cells after 6 h of incubation was evaluated by agarose gel (C). The fragments visualized were between 500 and 2,000 bp, with higher-molecular-weight fragments nonresolvable. Quantitation of soluble nucleosomal fragments by ELISA was based on cells incubated with anti-Fas or C_2-ceramide for 48 h or with camptothecin for 24 h (D). The variations within this experiment were 27.5 and 23.5% of the standard error of the mean for ceramide and camptothecin, respectively, and <3% for anti-Fas and the control. The difference between the control and both camptothecin and anti-Fas values was significant as determined by a two-tailed Student's *t* test ($P < 0.1$ and 0.001, respectively). The difference between the control and C_2-ceramide values did not achieve statistical significance as determined by a two-tailed *t* test.

acute diseases has been the subject of numerous reviews and monographs (30, 32, 34, 35). While the extracellular and intracellular stimuli which induce apoptosis differ, depending on the cell type, many of the morphologic and biochemical changes triggered during apoptosis appear similar. Chromatin condensation, reductions in overall cell volume, increases in granularity, endonuclease activation, nuclear fragmentation, and membrane blebbing have been reported in most studies of apoptosis.

Apoptosis plays a central role in the regulation of both the immunologic and the inflammatory responses, and aberrant regulation has been demonstrated to have catastrophic consequences in animal models. Defects in Fas antigen or Fas ligand in the *lpr* and *gld* mutations in mice, for example, result in autoimmune disease with autoantibodies similar to those in systemic lupus erythematosus (3, 8, 31, 33). An inability to induce apoptosis in these animals results in escape of autoreactive T cells from the thymus and massive lymphoproliferation within the spleen and lymph nodes. In contrast, human immunodeficiency virus infection of T cells and use of glucocorticoids result in excessive T-cell apoptosis and immunologic anergy (10, 18, 26, 32).

Apoptosis also represents an important regulatory process in myeloid cell function and senescence. Apoptosis in both neutrophil and monocyte cultures can be induced by removal of colony-stimulating factors and other cytokines (21, 22, 32). The role of macrophage clearance of apoptotic neutrophils as a mechanism by which inflammatory responses are minimized has been reviewed (28, 29). Apoptosis has also been reported to occur in macrophages with stimuli including gamma interferon (22) and following infection of the J774 macrophage cell line with *Shigella flexneri* (40). Intracellular infection with pathogenic organisms, however, does not uniformly result in macrophage apoptosis, as infection of macrophage colony-stimulating factor-depleted murine bone marrow macrophages with *Leishmania donovani* inhibited apoptosis (21). Therefore, in contrast to the uniform response of T cells to apoptotic inducers, including anti-CD3 and glucocorticoids, myeloid cell responses to apoptotic stimuli appear more variable, depending on the nature of the stimulus as well as the state of differentiation of the target cell.

The HL-60 promyelocytic leukemic cell line has served as a model for both granulocytic and monocytic differentiation (4). HL-60 and other myelogenous leukemic lines have also been useful in studying the effects of apoptotic stimuli on myeloid cells. Microtubule-disrupting drugs, including colchicine, vinblastine, nocodazole, and the calcium ionophore A23187, as well as protein kinase C inhibitors, DNA topoisomerase I and II inhibitors, and both protein and RNA synthesis inhibitors, have been demonstrated to induce apoptosis in this line (5, 9, 19, 20). Darzynkiewicz and coworkers, for example, have used this cell line to quantify camptothecin-induced apoptosis by flow cytometric techniques including PI staining, in situ labelling of DNA strand breaks, and acridine orange staining (5, 9, 13).

In the present study, apoptosis in HL-60 cells was investigated by using a combination of physiologic stimuli, namely, triggering of the Fas receptor by a monoclonal antibody against Fas antigen and use of cell-permeable ceramide analogs (6, 7, 14, 39). Apoptotic changes in nondifferentiated HL-60 cells were also investigated by means of a pharmaco-

logic stimulus by using the DNA topoisomerase I inhibitor camptothecin. Apoptosis with these three stimuli was both time and dose dependent, with maximal percentages of apoptotic cells evident by 48 h. While the morphologic assessment of apoptosis by these stimuli correlated with the appearance of internucleosomal fragments, flow cytometry permitted quantitation of the percentage of cells undergoing apoptosis at any point following addition of stimulus. Treatment of HL-60 cells with 5 μM C_2-ceramide, for example, resulted in >80% of the cells exhibiting apoptotic changes by 48 h (Fig. 2D). Therefore, the HL-60 cell line is a well-characterized myeloid cell line which is capable of a fairly uniform response by a majority of the cells to distinct apoptotic stimuli. This system lends itself to studies concerning the elements involved in the positive and negative regulation of apoptosis in response to external physiologic or pharmacologic stimuli.

Ceramide metabolism has been demonstrated to play an important role in the induction of myeloid differentiation of HL-60 cells mediated by tumor necrosis factor (TNF) interleukin 1 (12, 15, 16, 25) and can be mimicked directly by treating HL-60 cells with cell-permeable ceramide analogs (25). In addition to inducing differentiation, ceramide can induce apoptosis in both HL-60 (14) and U937 (24) cells and has been linked to the TNF signalling pathway through both ceramide-activated kinases and phosphatases (12, 16). However, the mechanism by which apoptosis is triggered remains unclear.

Induction of apoptosis through use of monoclonal antibodies against Fas antigen, a member of the TNF receptor family, has also been demonstrated in tumor lines as well as in T-cell cultures (7, 11, 38, 39). However, to what extent positive and negative regulators of apoptosis are able to independently modulate the cellular response to diverse apoptotic stimuli is not clear. Treatment of glioma lines with TNF and gamma interferon, for example, resulted in an increased sensitivity of these cells to undergoing apoptosis with anti-Fas antibodies (37). HL-60 offers the possibility, then, of investigating the effects of these as well as other inflammatory agents on the modulation of apoptosis in a well-characterized cell population. In addition to serving as a relatively homogeneous myeloid cell population for use in further studies of the molecular and biochemical events which occur during apoptosis, HL-60 should permit the identification of novel physiologic and pharmacologic agents capable of inducing the apoptotic program. The development of assays targeting apoptosis in HL-60 is an in vitro approach amenable to the identification of novel therapeutic agents designed to stimulate or inhibit apoptosis in myeloid cell populations. The efficacy of such therapeutic modalities will need to be evaluated in appropriate animal models simulating immunologic as well as both chronic and acute inflammatory diseases.

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