

Granulocytic differentiation of HL-60 cells results in spontaneous apoptosis mediated by increased caspase expression

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Abstract HL-60 cells differentiating into neutrophil-like cells die an apoptotic death in vitro. Susceptibility to apoptosis is associated with decreased Bcl-2 protein and mRNA expression; however, the effect of differentiation on the expression of pro-apoptotic caspases is unknown. Spontaneous apoptosis occurred 6 days after retinoic acid treatment. Western blotting showed loss of Bcl-2 by day 7, and new expression of ICE (caspase 1) and CPP32 (caspase 3) protein by day 2. Northern analysis demonstrated loss of Bcl-2 mRNA and increases in ICE mRNA by day 2; CPP32 mRNA was unchanged. Differential Bcl-2 and ICE mRNA expression was also found when granulocytic differentiation was stimulated by DMSO. Differentiated HL-60 cell lysates exhibited functional ICE proteolytic activity. De novo caspase expression was responsible for the development of spontaneous apoptosis, since specific inhibitors of ICE (YVAD-CMK) and CPP32 (DEVD-CHO), inhibited retinoic acid induced spontaneous apoptosis. Functional maturation and susceptibility to apoptosis are both inducible and linked in this granulocyte precursor cell line.

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Key words: Apoptosis; HL-60; Caspase; Bcl-2; Retinoic acid

1. Introduction

Mature blood neutrophils have a short life span both in vivo and in vitro. They die as a result of constitutive expression of apoptosis [1,2], a controlled biologic process leading efficiently to the death of the cell. Morphologic features of apoptosis include chromatin condensation, nuclear fragmentation associated with endonucleolytic DNA cleavage, cytoplasmic vacuolation, membrane blebbing and cell shrinkage, and ultimately the formation of apoptotic bodies which are removed by surrounding phagocytic cells [3]. Studies in the nematode, *C. elegans*, have revealed the presence of discrete genes that induce (*ced-3*, *ced-4*) [4] or block (*ced-9*) [5] expression of the apoptotic program. Human homologs of these have now been characterized. Bcl-2, the prototypic Ced-9 homolog in humans is a 26-kDa protein, originally identified in a B cell lymphoma, and known to be expressed in a variety of cells [6,7], that inhibits apoptosis and prolongs cell survival [8–11]. In contrast, the Ced-3 homologs, Interleukin 1 β converting enzyme (ICE or caspase-1 [12]) and CPP32 (caspase-3 [13]) are cysteine proteases, that induce apoptosis [4,13–15]. Caspases cleave substrates at aspartic acid residues, including

pro-interleukin 1 β [16,17], poly(ADP-ribose) polymerase [18] and the D4 GDP dissociation inhibitor, responsible for the regulation of Rho GTPase activity [19].

Cellular differentiation and cellular survival are functionally linked through altered expression of apoptosis. Thus promyelocytic HL-60 cells that are induced to differentiate into granulocytes express morphologic and functional features of mature neutrophils, and like neutrophils die a rapid apoptotic death [20]. The potential for transformed cells to be pharmacologically differentiated in vivo, and to undergo apoptosis provides a novel approach to antineoplastic therapy that has shown some preliminary efficacy in hematologic malignancies [21,22].

Mature neutrophils do not express Bcl-2 protein [23]. We therefore hypothesized that differential expression of pro- and anti-apoptotic genes underlies the process of constitutive neutrophil apoptosis. We show that the maturation of HL-60 cells into functionally active neutrophil-like cells involves a loss of Bcl-2 and new expression of active ICE and CPP32 protein, and results in the spontaneous expression of apoptosis in vitro. Specific inhibitors of ICE and CPP32 prevented the expression of spontaneous programmed cell death. Thus the acquisition of a constitutively expressed apoptotic program in HL-60 cells undergoing granulocytic differentiation is associated with, and dependent upon, the de novo expression of proapoptotic caspases, including caspase-1 (ICE) and caspase-3 (CPP32).

2. Material and methods

2.1. Reagents

RPMI, bicarbonate free RPMI 1640, penicillin and streptomycin solution, L-glutamine, phosphate buffered saline (PBS) and fetal calf serum (FCS) were purchased from GIBCO Life Technologies Ltd, Burlington, ON. YVAD-CMK, an irreversible inhibitor of ICE was purchased from Calbiochem, San Diego, CA. The CPP32 inhibitor, DEVD-CHO was purchased from Biomol, Plymouth Meeting, PA. All other chemicals were supplied by SIGMA Chemical Co., St. Louis, MO, unless otherwise stated. Dihydrorhodamine 123, N-methyl-D-glucammonium (NMG), nigericin, monensin, and the acetoxymethyl ester form of 2',7'-bis-carboxyethyl-5-(6)-carboxyfluorescein (BCECF) were purchased from Molecular Probes, Eugene, OR.

2.2. Antibodies

FITC-labeled mouse anti-human CD11b (Clone D12, IgG_{2a}) and FITC-labeled mouse anti-human CD14 (Clone MØ-P9, IgG_{2b}) were supplied by Becton Dickinson, San Jose, CA. Mouse anti-human Bcl-2 oncoprotein (Clone 124, IgG1) was supplied by DAKO, Carpinteria, CA. Rabbit anti-human ICE polyclonal antibody (Clone AP-1, IgG) was supplied by Calbiochem, Cambridge, MA. Mouse anti-human CPP32 monoclonal antibody (Clone 19, IgG_{2a}) was supplied by Transduction Laboratories (Bio/Can Scientific), Mississauga, ON. Anti-Fas antibody (Clone CH-11) and FITC labeled UB2 mouse anti-human Fas were supplied by Immunotech, Marseille, France.

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2.3. Probes

Human Bcl-2 cDNA probe was supplied by Calbiochem, Cambridge, MA. Human ICE cDNA was kindly donated by Immunex, Seattle, WA. CPP32 probe was kindly supplied by Dr. V.M. Dixit, University of Michigan Medical School, MI.

2.4. Cells

HL-60 cells (American Type Culture Collection, Rockville, MD), were maintained in suspension in RPMI supplemented with 10% FCS. Granulocytic maturation was induced with 1 μ M all trans retinoic acid or DMSO (1.25%) [23]. Cell numbers were determined in a hemocytometer chamber; cell viability was assessed by trypan blue dye exclusion.

Neutrophils were isolated from healthy volunteers by dextran sedimentation and centrifugation through a discontinuous ficoll gradient [24]. The PMN pellet was resuspended at a concentration of 1×10^6 cells/ml in DMEM supplemented with 10% fetal calf serum, 1% glutamine and 1% penicillin/streptomycin solution. Cells were incubated in polypropylene tubes to prevent adherence. PMN purity as assessed by size and granularity on flow cytometry was consistently greater than 95%.

2.5. Western blot analysis

For SDS-PAGE of whole cell extracts, 2×10^6 human neutrophils or HL-60 cells were sedimented and run on a 12% SDS polyacrylamide gradient gel, then electrophoretically transferred to Immobilon-P (Millipore, Bedford, MA). Blots were incubated with primary antibody in 1% BSA TBS and 0.1% Tween 20 for 1 h at room temperature and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG at 1:5000 dilution for 1 h. Blots were developed using an enhanced chemiluminescence system [25].

2.6. Northern blot analysis

Total RNA was extracted from human neutrophils (50×10^6) or HL-60 cells (20×10^6) using the guanidium-isothiocyanate method [26]. RNA was denatured, electrophoresed through a 1.2% formaldehyde-agarose gel and transferred to nylon membrane. Hybridization was performed at 42°C for 18 h with a [α - 32 P]dCTP-labeled, random-primed cDNA probe for either human ICE, CPP32 cDNA or human Bcl-2 cDNA. Membranes were stripped and hybridized to an 18s ribosomal subunit cDNA probe; mRNA expression was quantitated using a phosphorimager with accompanying ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The mRNA signal was standardized to the 18s RNA signal to correct for variability in gel loading.

2.7. DNA gel electrophoresis

DNA laddering for assessment of HL-60 DNA fragmentation was carried out as previously described [24].

2.8. Quantification of apoptosis

Human neutrophil and HL-60 cell apoptosis was quantitated as the percent of cells with hypodiploid DNA using the technique of Nicoletti et al [27]. Cell suspensions were centrifuged at $200 \times g$ for 10 min. The cell pellets were gently resuspended in 1 ml of hypotonic fluorochrome solution (50 μ g/ml PI, 3.4 mM sodium citrate, 1 mM Tris, 0.1 mM EDTA, 0.1% Triton X-100) and stored in the dark at 4°C for 3–4 h before they were analyzed using a Coulter EPICS XL-MCL cytofluorometer. A minimum of 5000 events were collected and analyzed. Apoptotic PMN nuclei were distinguished from normal PMN nuclei by their hypodiploid DNA.

2.9. Surface receptor expression

HL-60 cells (1×10^5 /100 μ l) were stained with 10 μ l of the appropriate FITC labeled monoclonal antibody for 20 min at 4°C. Cells were then washed twice in cold PBS and analyzed by flow cytometry.

2.10. Respiratory burst

Whole cell reactive oxygen intermediates were quantitated using the technique of Smith et al [28]. HL-60 cells (1×10^5 /ml) from each of the different groups were incubated with 1 μ M dihydrorhodamine 123 for 5 min at 37°C and then stimulated with PMA (5 nM) for 10 min. Fluorescence intensity was assessed by flow cytometry and expressed as Ln mean channel fluorescence (LnMCF).

2.11. Cytosolic pH measurement

Spectrofluorimetry was used for continuous monitoring of cytosolic pH in cells loaded with the pH-sensitive fluorescent probe BCECF as previously described [29].

2.12. Protease activity assay

Cell lysates were prepared from 10×10^6 HL-60 cells after different treatments. Aliquots of the lysates (10 μ l) were diluted in assay buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS) containing 20 μ M Ac-Tyr-Val-Ala-Asp-AMC (Calbiochem, Hornby, ON), and

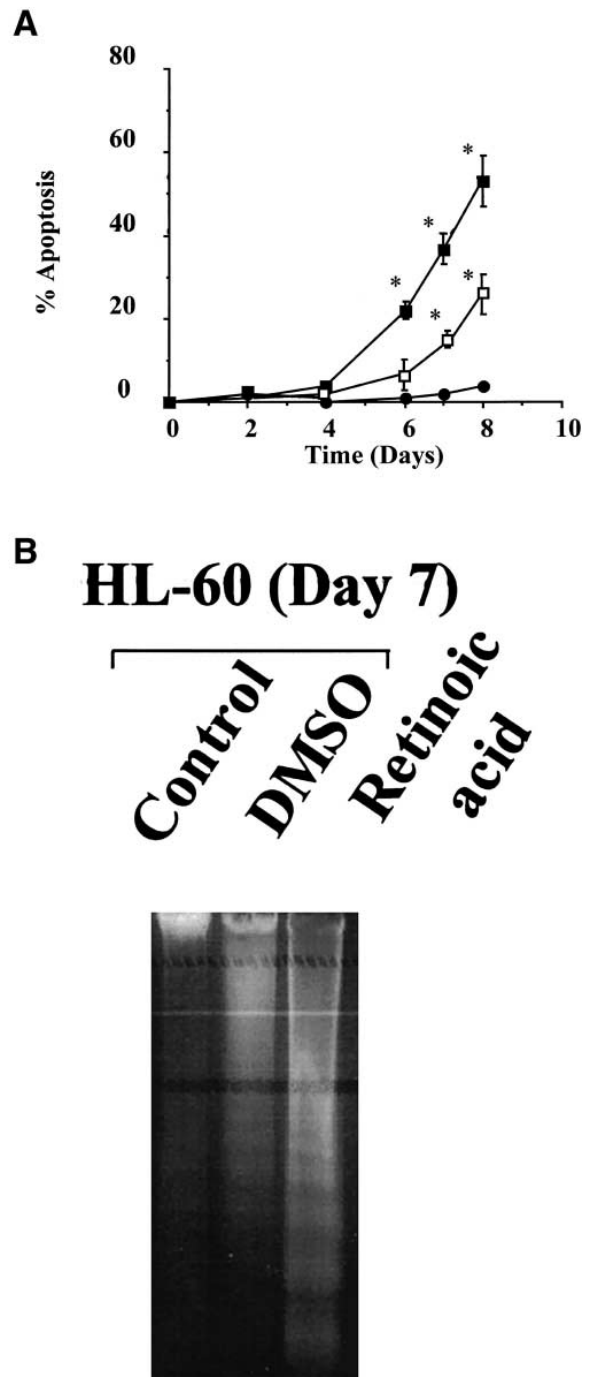


Fig. 1. Spontaneous apoptosis in HL-60 cells differentiated with retinoic acid (1 μ M) or DMSO (1.25%). Apoptosis was assessed by propidium iodide DNA staining (A) ($p < 0.05$, $n = 8$) and by DNA gel electrophoresis (B). DNA gel is representative of one of five experiments. * $p < 0.05$ vs Control HL-60. Control (closed circles), retinoic acid (1 μ M) (closed square) and DMSO (1.25%) (open square).

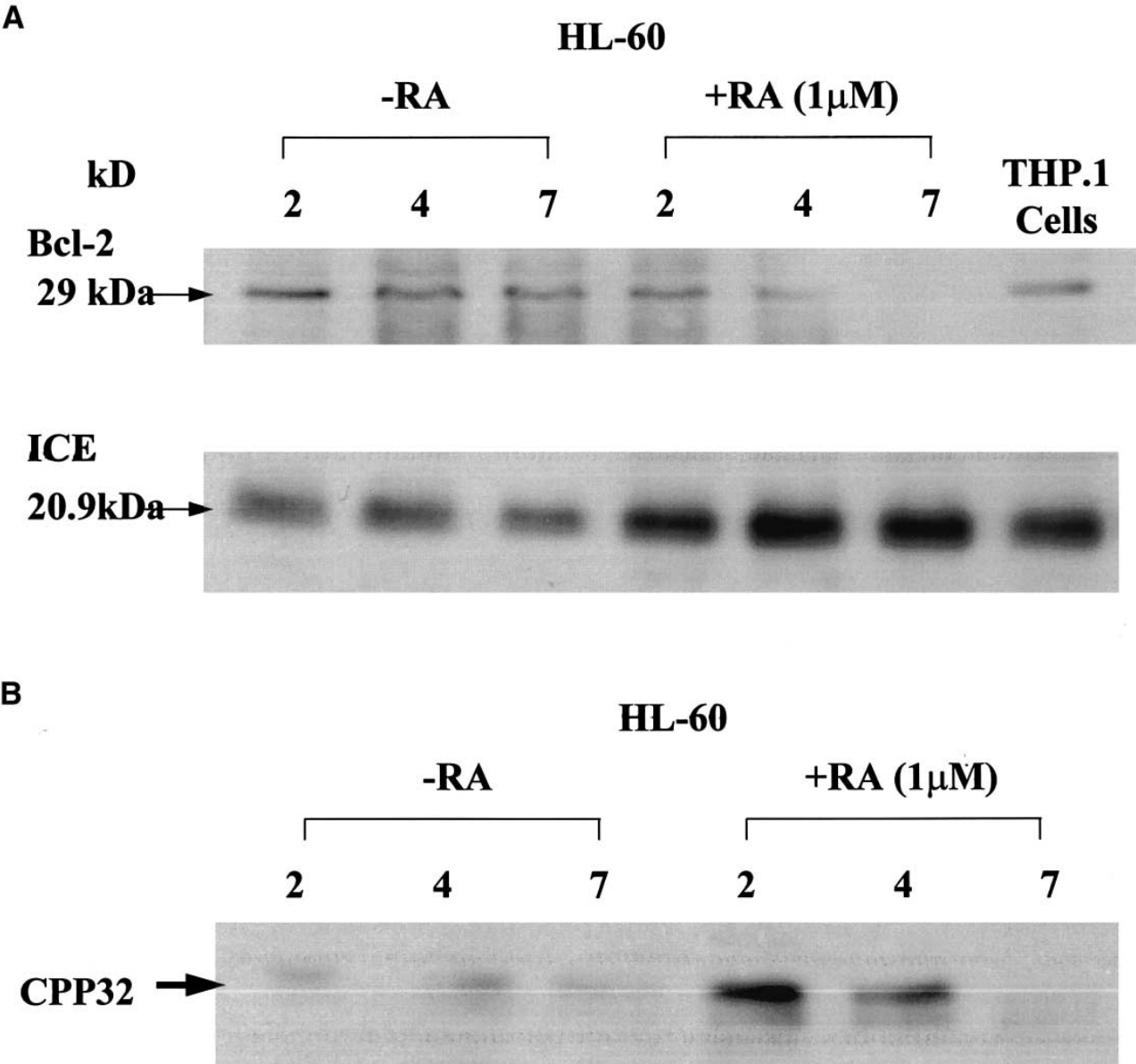


Fig. 2. Bcl-2, ICE and CPP32 protein expression in retinoic acid differentiated HL-60 cells. Protein was extracted at the times indicated to assess Bcl-2 (A), ICE (A) and CPP32 (B) protein expression. THP.1 cells served as positive controls for Bcl-2 and ICE. Results are representative of one of three separate experiments.

incubated for 45 min at room temperature. The release of AMC was detected by continuous measurement in a luminescence spectrometer (Perkin Elmer LS50, London, England) with an excitation of 380 nm and an emission slit at 460 nm. Specific activity is measured as pmol s⁻¹ per mg protein

2.13. Statistics

Statistical analysis was carried out using ANOVA with Scheffé correction. Significance was assumed for values of *p* < 0.05.

Table 1
Effects of retinoic acid induced maturation on HL-60 cell functional activity

	Days of retinoic acid (1 μM) treatment			
	0	2	4	6
CD11b expression (LnMCF)	10 ± 5	12 ± 9	32 ± 16*	49 ± 12*
CD14 expression (LnMCF)	5 ± 2	162 ± 5	20 ± 9*	23 ± 6*
Respiratory Burst (LnMCF)	20 ± 11	25 ± 9	46 ± 20*	65 ± 15*
pH Recovery (pH/min)	0.20 ± 0.04	0.22 ± 0.05	0.41 ± 0.03*	0.59 ± 0.03*

HL-60 cells cultured with retinoic acid over 6 days were assessed for the above.

**p* < 0.05 vs day 0, *n* = 5.

3. Results and discussion

3.1. Granulocytic maturation of HL-60 cells results in neutrophil-like functional activity

Treatment of HL-60 cells with either retinoic acid or DMSO has been shown to result in their differentiation into neutrophil-like cells, with morphologic features of mature neutrophils [22,30]. Treatment of HL-60 cells with 1 μ M retinoic acid for 5 days resulted in neutrophil-like functional activity, manifested in increased respiratory burst in response to PMA (5 nM) and augmented recovery from an acid load

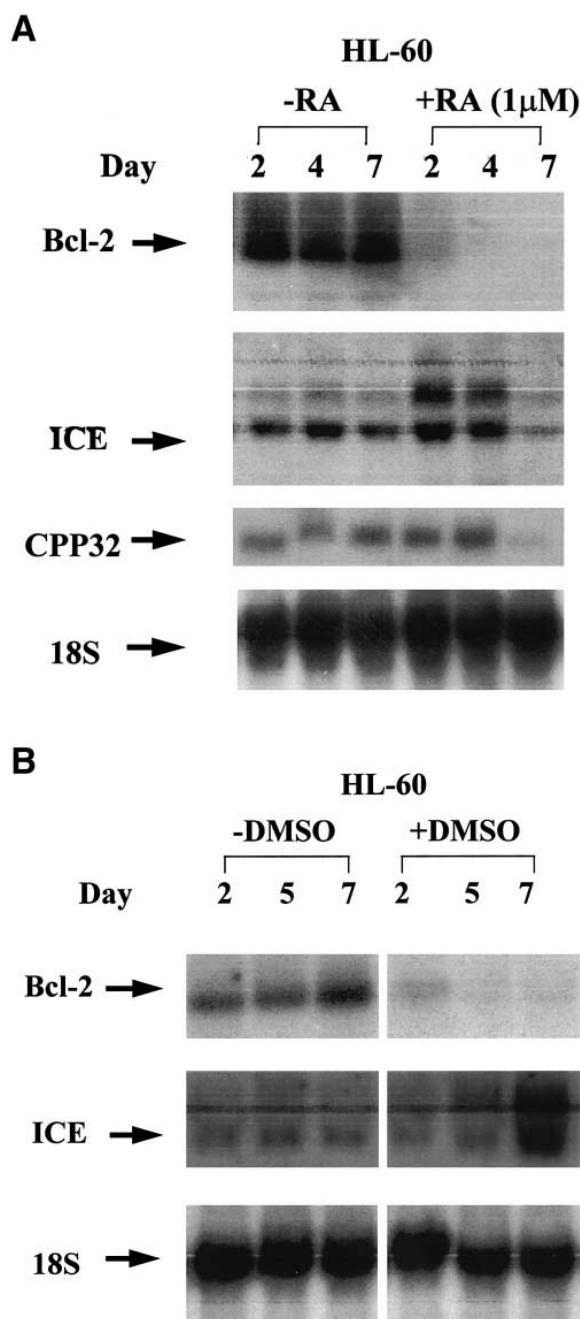


Fig. 3. Bcl-2, ICE and CPP32 mRNA in (A) retinoic acid and (B) DMSO differentiated HL-60 cells. Total RNA was extracted after incubation for the times indicated. Northern blots were probed for 18S to ensure equal loading. Blot is representative one of four experiments.

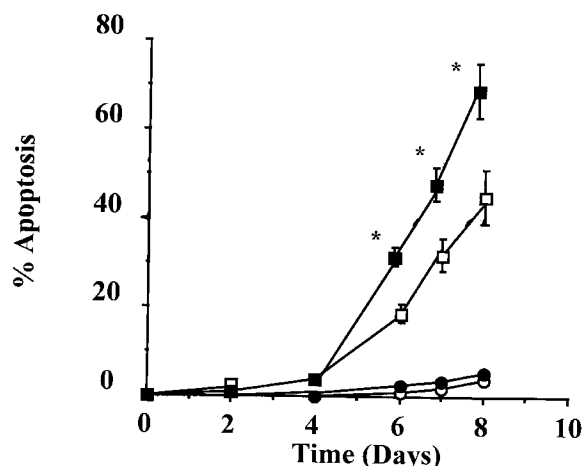


Fig. 4. Effects of anti-Fas monoclonal antibody (CH-11) on apoptosis of immature and retinoic acid matured HL-60 cells. Cells were harvested after 5 days and then incubated with or without anti-Fas antibody (CH-11, 100 ng/ml). Apoptosis was assessed by flow cytometry on the days indicated. * $p < 0.05$ vs control HL-60 cells. Control (open circle), Control+Anti-Fas antibody (100 ng/ml) (closed circle), Retinoic acid (1 μ M) (open square) and Retinoic acid (1 μ M)+Anti-Fas antibody (100 ng/ml) (closed square).

(Table 1), as well as increased expression of surface receptors CD11b and CD14 (Table 1).

3.2. Maturing HL-60 cells undergo spontaneous apoptosis

While undifferentiated HL-60 cells survive indefinitely in culture, differentiated HL-60 cells began to undergo spontaneous apoptotic cell death, starting on day 4 after retinoic acid treatment and on day 7 following DMSO (1.25%) treatment (Fig. 1a). DNA gel electrophoresis (Fig. 1b) demonstrated the characteristic DNA laddering of apoptosis. Martin et al have previously demonstrated that HL-60 cells die by apoptosis after 5 days of culture with retinoic acid, and that cells with apoptotic morphology only appear in appreciable numbers when cells with mature neutrophil phenotypes are the predominant cell type [22].

3.3. Alterations in Bcl-2 and ICE protein and mRNA on HL-60 differentiation

Retinoic acid differentiation of HL-60 cells resulted in a time-dependent decrease in Bcl-2 protein expression (Fig. 2), similar to that occurring when HL-60 cells are matured with TPA or DMSO [31]. ICE is synthesized as a 42-kDa precursor that is cleaved by ICE-like proteases to yield an active 20-kDa protein. Maturing HL-60 cells showed increased expression of the 20-kDa pro-apoptotic ICE protein (Fig. 2a). CPP32 protein, another member of the caspase family of proteases [13], was also significantly increased after retinoic acid treatment of HL-60 cells (Fig. 2b). Expression of both ICE and CPP32 occurred during the first 2 days of granulocytic differentiation.

Bcl-2 mRNA was significantly decreased as early as day 2 (Fig. 3a). Retinoic acid treatment resulted in early increases in ICE mRNA (Fig. 3a), declining again by day 7, at which time over 40% of the cells expressed apoptotic characteristics. There were no alterations in CPP32 mRNA after treatment with retinoic acid (Fig. 3a) although CPP32 protein was increased.

Changes in mRNA expression were a consequence of the

differentiation of HL-60 cells, since comparable changes in Bcl-2 and ICE expression occurred in cells matured with DMSO (1.25%) (Fig. 3b), although maturation was slower, and the increase in spontaneous apoptosis delayed (Fig. 1a) compared to retinoic acid.

Interleukin 1 β converting enzyme proteolytic activity was measured in cell lysates after 4 days of retinoic acid treatment. AMC release was greater in differentiated HL-60 cell lysates (96 ± 10 pmol s $^{-1}$ per mg protein) compared to control HL-60 cell lysates (21 ± 12 pmol s $^{-1}$ per mg protein) ($p < 0.05$), demonstrating increased caspase proteolytic activity, coincident with the acquisition of a constitutively apoptotic phenotype. Previous studies have demonstrated that anisomycin and geranylgeraniol induce HL-60 cell apoptosis through increased processing and activation of caspase 3 (CPP32) [32], and that Topoisomerase II directed against etoposide induces the activation of CPP32, ICE and caspase 6 in apoptotic HL-60 cells [33]. Moreover, etoposide-induced apoptosis in HL-60 cells can be blocked by an ICE-like protease inhibitor, Z-Val-Ala-Asp-fluoromethyl ketone [34]. Thus the caspase family of proteases has been implicated in the induction of apoptosis in HL-60 cells in response to exogenous stimuli. We now show a role for newly synthesized caspases in the susceptibility to spontaneous or constitutively expressed apoptosis that develops during HL-60 granulocytic differentiation.

3.4. Maturing HL-60 cells acquire sensitivity to Fas-mediated death signals

Fas (APO-1, CD95), a 45-kDa type I membrane protein signals for apoptotic cell death [35] and is expressed on human neutrophils [36]. Fas was also shown to be expressed in immature HL-60 cells at levels comparable to those found on mature neutrophils (7.9 ± 3.8 LnMCF and 9.4 ± 2.9 LnMCF,

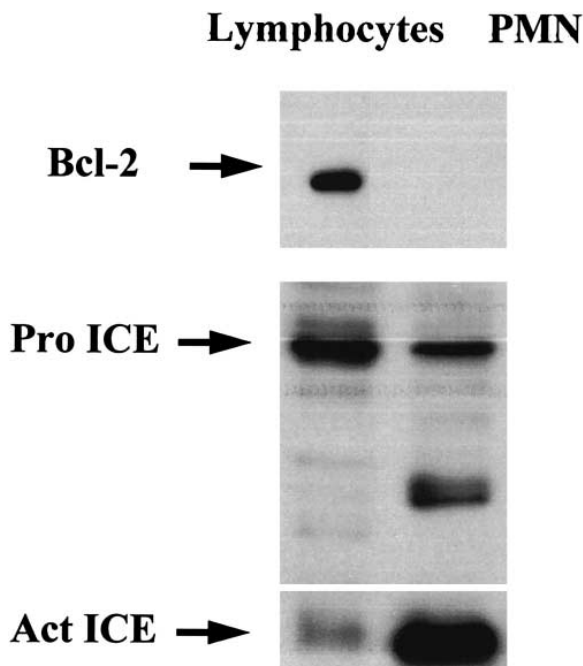


Fig. 5. Bcl-2 and ICE protein expression in human neutrophils. Western blotting of protein from freshly isolated neutrophils shows both proICE (42 kDa) and the mature form (20 kDa) of ICE. Bcl-2 was evident in lymphocytes, but not mature neutrophils. Blot represents one of three experiments.

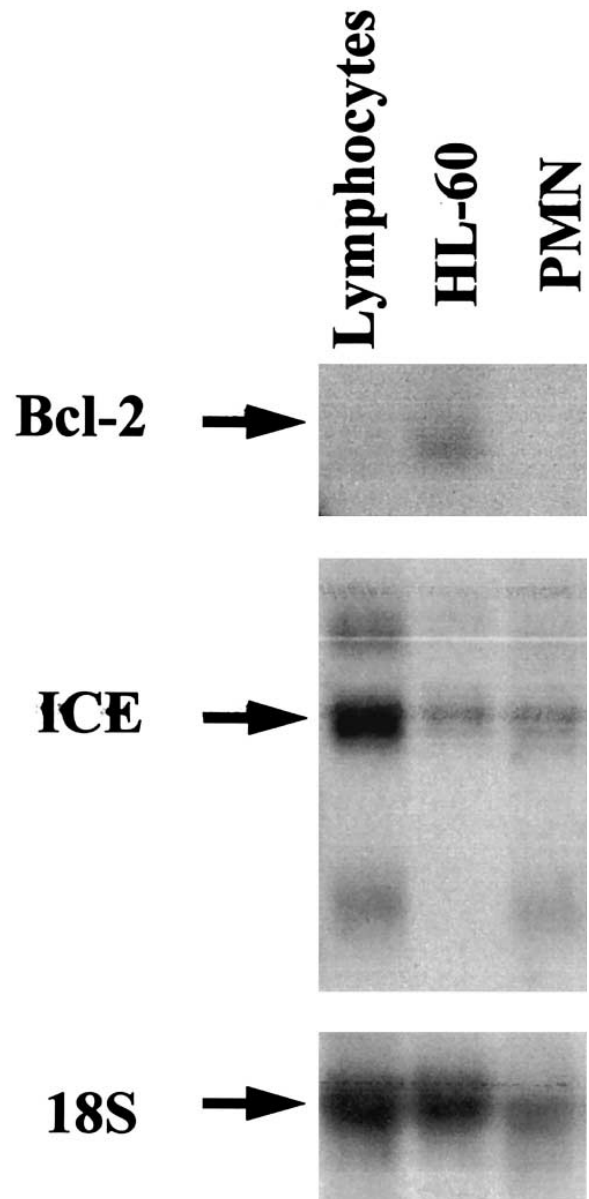


Fig. 6. Bcl-2 and ICE mRNA in mature human neutrophils. Total RNA was extracted from neutrophils, immature HL-60 cells and lymphocytes. Blot represents the results of three separate experiments.

respectively). Fas expression was not altered by differentiation with retinoic acid (6.5 ± 4.1 LnMCF). Immature HL-60 cells have been shown to be resistant to anti-Fas antibody induced apoptosis [37]. Retinoic acid differentiated HL-60 cells incubated with anti-Fas monoclonal antibody, CH-11, (100 ng/ml) on day 5 showed a significant increase in rates of apoptosis (Fig. 4) as compared to immature HL-60 cells. Since the caspase proteases, ICE and CPP32, mediate Fas signaled apoptosis [38,13], susceptibility to Fas-induced death signals may be a consequence of their de novo expression.

3.5. Apoptotic gene expression in mature HL-60 cells mirrors that of neutrophils

Both Bcl-2 [19] and Bcl-x [39] are reported to be absent in mature neutrophils. We found that although mature neutro-

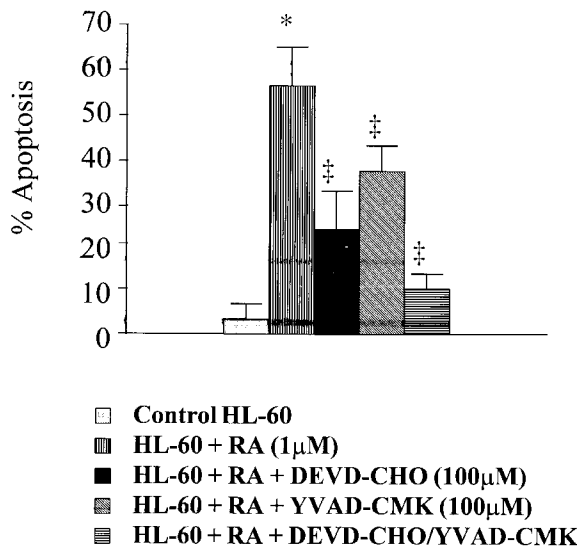


Fig. 7. Effects of specific caspase inhibitors on spontaneous apoptosis of retinoic acid matured HL-60 cells. HL-60 cells were matured with retinoic acid (1 µM) for 5 days, then incubated with or without the ICE inhibitor, YVAD-CMK or CPP32 inhibitor, DEVD-CHO alone or in combination. $n=4$; * $p<0.05$ vs control HL-60, † $p<0.05$ vs HL-60+RA.

phils do not express Bcl-2 protein, they have high levels of both the pro- and active forms of ICE protein (Fig. 5). Similarly, Northern blot analysis of mature neutrophils demonstrated no Bcl-2 mRNA, although ICE mRNA was present (Fig. 6).

3.6. Inhibition of ICE partially inhibits the spontaneous apoptosis of retinoic acid matured HL-60 cells

Caspases have been implicated in etoposide induced HL-60 cell apoptosis [32–34]. To establish the role of ICE and CPP32 in the induction of spontaneous apoptosis in mature HL-60 cells, the irreversible ICE inhibitor, Ac-Tyr-Val-Ala-Asp-CMK (YVAD-CMK) (100 µM) and the specific CPP32 inhibitor *N*-acetyl-Asp-Glu-Val-Asp-CHO (DEVD-CHO) (100 µM) were added to maturing HL-60 cells on day 5. These inhibitors reduced, but did not completely prevent spontaneous apoptosis when used alone; however, in combination there was nearly total prevention of spontaneous apoptosis (Fig. 7). YVAD-CMK and DEVD-CHO had no effect on immature HL-60 cell viability. ICE-like inhibitors not only block differentiation induced HL-60 cell spontaneous apoptosis but *Z*-Val-Ala-Asp-fluoromethyl ketone also inhibits etoposide [37], anisomycin and geranylgeraniol [38] induced apoptosis in immature cells.

Cell growth and differentiation occur through differential gene expression. Retinoic acid induced maturation of HL-60 cells results in the expression of both neutrophil-like functional activity and susceptibility to spontaneous cell death, through the controlled process of apoptosis. The development of this state of constitutive apoptosis is associated with suppression of Bcl-2 and activation of the pro-apoptotic proteases ICE and CPP32. HL-60 cells are a useful model for examining the molecular mechanisms involved in the regulation of spontaneous apoptosis in hematopoietic cells, and, in particular, for further elucidating the role of interactions be-

tween pro- and anti-apoptotic intracellular cascades in the regulation of cell survival.

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