# Ethanol Induced Apoptosis in Human HL-60 Cells

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**Abstract.** In this study flow cytometric and morphologic methods of apoptosis detection in human promyelocytic leukemia cell line HL-60 were compared. HL-60 cells were harvested at 4, 7, 16, 24 a 48 hours after induction of apoptosis by 3 %ethanol. Little changes were observed both by flow cytometry (decrease of forward scatter, increase of unprocessed cells staining with APO2.7 antibody) and viability determination by Trypan-blue staining until after 7 hours. However, after 4 hours morphologic changes were observed in the nuclear and cytoplasmic structures using Diff-Quik stained cytospin preparations and standard light microscopic techniques (50% apoptotic cells). The same results were obtained by flow cytometric measurement of sub-diploid DNA content (sub- $G_1$  cells), and an increase of staining with APO2.7 antibody in cells permeabilised by digitonin prior to staining. After 7 hours almost all cells exhibited apoptotic morphology. After 16 hours the cell size (forward scatter) decreased significantly, and 54% of unprocessed cells were APO2.7 positive. After 24 hours only 6% of cells were alive (high forward scatter) and these cells were APO2.7 negative. The HL-60 cells did not proliferate during the cultivation in 3% ethanol, and after 48 hours all stained by Trypan blue. HL-60 leukemic cells were  $CD34^-/AC133^-$ ,  $CD33^+/CD15^+$ , and only 2% of the cells were CD95<sup>+</sup>. Induction of apoptosis by ethanol did not enhance CD95 antigen expression.

Key words: Apoptosis — Ethanol — HL-60 cells

### Introduction

Programmed cell death (apoptosis) is a genetically defined pathway of cellular suicide required for embryonal development, growth and differentiation of multi-

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cellular organisms as well as for tissue homeostasis. In addition, it serves as one of the pleiotropic mechanisms of cell killing by cytokines and cytotoxic T-cells and appears to be the basic mechanism underlying the anti-oncogenic effect of most chemotherapeutic drugs and X-ray treatment (Herr et al. 1997).

Apoptotic dying cells exhibit characteristic changes, including chromatin condensation, formation of 'apoptotic bodies', membrane blebbing and endonuclease degradation of chromosomal DNA into nucleosome-sized pieces of about 180 base pairs. Normally, no single method is sufficient to identify apoptotic cell death. Methods to identify cells undergoing apoptotic cell death have been devised to detect DNA strand breaks in individual cells (Li and Darzynkiewicz 1995), phospholipid membrane asymmetry and exposure of phosphatidylserine using Annexin V (Koopman et al. 1994), DNA fragment laddering by gel electrophoresis (Duke et al. 1983), morphology using light (Nagata and Golstein 1995) and light scattering changes by flow cytometry (Ormerod et al. 1995). One morphological marker associated with apoptosis is the loss of cellular volume in response to cytoplasmic condensation that is associated with internucleosomal DNA fragmentation, a well described event in apoptosis. The classical events of apoptosis may not always occur. Apoptosis without the typical DNA fragmentation (Ormerod et al. 1994; Schulze-Osthoff et al. 1994) has been reported, as well as an experimental model of apoptosis in cells without nuclei (Schulze-Osthoff et al. 1994).

Evidence continues to build up in support of early physiological events that may be shared by cells undergoing apoptotic death. More recently, the involvement of mitochondria in apoptotic signaling has elicited considerable interest. Cells are reported to undergo a reduction of mitochondrial transmembrane potential before exhibiting the classic signs of apoptosis (Herkart and Grinstein 1996). Cells having lost their mitochondrial transmembrane potential, through the opening of mitochondrial permeability transition pores, appear to be irreversibly programmed to die (Marchetti et al. 1996). Koester et al. (1997) used APO2.7 (anti-7A6) monoclonal antibody which reacts preferentially with a 38-kDa mitochondrial membrane protein in cells undergoing apoptosis for monitoring early apoptotic responses in anti-CD95 (7C11)-treated Jurkat cells. Unprocessed cells stained with APO2.7 antibody showed little increase in staining until after 6 h following induction of apoptosis, when DNA fragmentation was demonstrated by flow cytometry and gel electrophoresis; however processed cells stained with APO2.7 antibody showed significant increase in staining after 1.5 h. APO2.7 is a useful marker for detection of apoptotic cells. Data presented by Koester et al. (1997) indicate that the antigen identified by APO2.7 appears early in the apoptotic process and thus, when cells are permeabilised prior to staining, this antigen can be identified. It is possible to compare the staining profile ratios of permeabilised to nonpermeabilised cells to monitor early and late apoptotic events in a simultaneous dual staining assay.

The outcome of chronic ethanol consumption recorded in the liver by *in situ* staining of the genomic DNA in fragmented nuclei indicates the course of cellular events that has been coined as apoptosis. Isolated hepatocytes derived from alcohol-consuming rats contained 2–4 fold higher amounts of nucleosomal fragments in the

cytosolic extracts in comparison with control rats. The association between the chronic consumption of ethanol and increased susceptibility to infections is well-recognized (Pavia et al. 1991). The enhanced apoptosis of monocytes/macrophages is one potential mechanism that may lead to ethanol-induced loss of leukocytes and contribute to the increased susceptibility to bacterial infections and cancer, which is associated with chronic ethanol abuse. Aroor and Baker (1997) observed characteristic features of apoptosis after 4 days 100 mmol/l (0.5%) ethanol treatment of HL-60 cells, as documented by increased DNA fragmentation and enhanced expression of phosphatidylserine, an early marker of apoptosis.

The HL-60 human promyelocytic leukemia cell line has been well characterized as an in vitro model for cell proliferation, differentiation and apoptosis (Kravtsov and Fabian 1996; Hofmanová et al. 1998). In this study, we investigated whether ethanol in concentrations 0.5-4% induced apoptosis and loss of HL-60 cells during 48 hours of incubation. Within this time range, a decrease of cell proliferation (ethanol concentrations 1-4%) and apoptotic cell death (ethanol concentrations 2-4%) were observed. Incubation of HL-60 cells with 0.5% ethanol for 48 hours did not increase the percentages of detected apoptotic cells and did not significantly decrease cell proliferation.

### Materials and Methods

#### Cell culture and culture conditions

Human leukemia HL-60 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK) and were cultured in MyeloCult H5100 medium (StemCell Technologies Inc., Vancouver, Canada) in a humidified incubator at 37 °C and controlled 5% CO<sub>2</sub> atmosphere. The cultures were divided every 3<sup>rd</sup> day by dilution to a concentration of  $2 \times 10^5$  cells/ml. Cell counts were performed with a hemocytometer, cell membrane integrity was determined using the Trypan blue exclusion technique. HL-60 cells in the maximal range of 20 passages were used for this study.

# $Cell\ treatments$

Exponentially growing HL-60 cells were suspended at a concentration of  $2 \times 10^5$  cells/ml in complete medium. 10 ml of aliquots were plated into 25 cm<sup>2</sup> flasks (Nunc) and mixed with ethanol at desired concentrations. Following 4, 7, 16, 24 and 48 hours the cells were counted and cell viability was determined with the Trypan blue exclusion assay.

#### Cell morphology

To calculate the percentage of cells showing morphology of apoptosis, cell aliquots were removed from control and drug-treated cell cultures at various times of incubation and usually 400 cells were counted on Diff-Quik (Dade Behring, Switzerland) stained cytospin preparations. Apoptotic cells were identified by the condensed and fragmented state of their nuclei and focal protrusions of the cell surface.

#### Cell surface markers and cell size analysis

Flow cytometry was used for cell surface antigen analysis and also for monitoring apoptosis. Cells were washed twice with PBS containing 5% FCS. Then,  $1 \times 10^5$  cells suspended in 0.5 ml PBS with 5% FCS and 0.02% NaN<sub>3</sub> were incubated with mAbs for 30 min at 4°C.

For apoptosis detection the mouse phycoerythrin (PE)-conjugated mAb APO2.7 (clone 2.7 A6A3) (obtained from Immunotech, Prague, CR) for detecting 7A6 antigen expressed by cells undergoing apoptosis was used. Nonpermeabilised and permeabilised method was used. Cells were permeabilised in 100  $\mu$ l of 100  $\mu$ g/ml digitonin solution in PBS and incubated for 20 min on ice. Cells were washed and stained with APO2.7.

For detection of cell surface markers we used fluorescein isothiocyanate (FITC)conjugated mouse anti-human CD34, and CD15 mAbs and PE-conjugated antihuman CD33 and CD95 mAbs (obtained from Immunotech) and anti-AC-133-PE (from Miltenyi Biotec, Germany).

The ability of the cells to scatter light in a forward direction (FS) correlates with cell volume. We analyzed ethanol-treated cells for changes in the intensity of FS as compared to untreated control cells. Flow cytometric analysis was performed on a Coulter Epics XL flow cytometer equipped with a 15mW argon-ion laser with excitation capabilities at 488 nm (Coulter Electronic, Hialeah, FL, USA). A minimum of 10,000 cells was collected for each 2-colour sample in a list mode file format. List mode data were analyzed using Epics XL System II software (Coulter Electronic, Hialeah, FL, USA).

# Cell cycle analysis

Following 4, 7, 16, 24 and 48h of incubation, the cells were washed with cold PBS, fixed by 70% ethanol and stained with propidium iodide (PI) in Vindelov's solution for 30 minutes at 37 °C. Fluorescence (DNA content) was measured with Coulter Electronic (Hialeah, FL, USA) apparatus. A minimum of 10,000 cells analyzed in each sample served to determine the percentages of cells in each phase of the cell cycle, using Multicycle AV software. Three independent experiments were performed.

### Statistical analysis

The results were statistically evaluated with Student's t-test. The value represent mean  $\pm$  SD (standard deviation of the mean) of three independent experiments. Statistical significance of the differences of means between comparable sets is indicated.

#### Results

# Antigen expression

We described antigen expression on HL-60 cells obtained from the European Collection of Animal Cell Cultures. The cells were negative for the expression of CD34 and AC133 antigens (0.6/0.7%), which are usually used for separation of hematological progenitors from mobilized peripheral blood patients for autologous transplantation. HL-60 cells expressed high levels of CD15 (93%) and CD33 (84%) antigens, 82% cells were CD15<sup>+</sup>/CD33<sup>+</sup> (Fig. 1). Hematopoietic progenitor cells giving rise to monocytic and granulocytic lineages express numerous surface antigens to varying degrees depending on their developmental stage: CD33 antigen is expressed prior to myeloid commitment and CD15 is expressed at later stages in myelomonocytic development. Hofmanová et al. (1998) reported 90% HL-60 cells being promyelocytes and 8% myelocytes with no expression of CD14/CD11b antigens. Only 2% of HL-60 cells expressed CD95 antigen.



Figure 1. Antigen expression of HL-60 cells. PE versus FITC fluorescence intensity (CD33/CD15, AC133/CD34) and PE fluorescence intensity (CD95) characteristic plots are shown. A minimum of 10,000 cells were collected.

# Cell growth and viability

Fig. 2 shows the effects of ethanol on the proliferative rate of the HL-60 cell line. Cultivation with 0.5% ethanol had no effect on the proliferation of HL-60 cells. Cultivation with 1% ethanol induced a slight decrease of proliferation in comparison with control cells. Cultivation with 2% ethanol induced high inhibition of the rate of HL-60 cell growth. The decrease of the proliferative rate observed in HL-60 cells after addition of 3 or 4% ethanol was due to the cytotoxic effect of ethanol. After 48 hours all cells treated with 3 and 4% ethanol were dead.



Figure 2. Kinetics of the ethanol effect on the proliferative rate of the HL-60 cell line. HL-60 cells were exposed to various ethanol concentrations (0.5-4%). Numbers of viable cells were determined by Trypan blue staining.

# Morphologic changes

HL-60 cells were incubated in the presence 0.5; 1; 2; 3 or 4% ethanol for 48 hours. After 1, 4, 7, 24 and 48 hours cell morphology was examined on Diff-Quik stained cytospin preparations. Time and ethanol dose dependent increase in the proportion of apoptotic cells was detected in cultures exposed to ethanol. Four hours after addition of ethanol an increase in the proportions of apoptotic cells was detected in cultures treated with 2–4% ethanol. The highest percentages of apoptotic cells were observed in cultures incubated for 7 hours with 3 and 4% ethanol (Fig. 3).

### Analysis of cell size

Changes in cell size in response to 3% ethanol were analyzed by flow cytometry. The ability of cells to scatter light in a forward direction (FS) correlates with cell volume. A cell triggered to undergo apoptosis activates a cascade of molecular events, which leads to its total disintegration. One of the early events is condensation of the cytoplasm followed by a change in cell size and shape. Therefore, we analyzed ethanol treated cells for changes in the intensity of FS as compared to untreated control. Our results showed that exposure of HL-60 cells to 3% ethanol resulted in a decrease in FS by flow cytometry with the increasing time (Fig. 4), indicating cell shrinkage. After 24 hours practically all cells incubated with 3% ethanol showed low FS (82 %).



Figure 3. Time course of apoptosis in HL-60 cells exposed to ethanol as determined by cell morphology examined on Diff-Quik stained cytospin preparations.

### Analysis of sub-diploid DNA content

We assessed DNA cleavage in the aforementioned ethanol-treated tumor cells by immediately fixing cells in ethanol, followed by PI staining of DNA content. Apoptotic cells exhibit chromatin condensation and form 'apoptotic bodies' and therefore can be recognized following staining of cellular DNA as cells with low DNA stainability (sub-G<sub>1</sub>). As early as 4 hours after exposition to 3% ethanol the number of sub-G<sub>1</sub> cells increased to 50% while the number of cells in all other cell-cycle phases decreased (Fig. 5). The percentages of apoptotic cells and cells in other cell-cycle phases after incubation with 3% ethanol are summarized in Table 1. During the 48 hours of incubation the decrease of the numbers of cells in cell-cycle diminished until after 48 hours when these cells were not present at all. Simultaneously the numbers of apoptotic cells increased and only this type of cells was present after 48 hours.

## Flow cytometric detection of apoptotic cells using monoclonal antibody APO2.7

A novel monoclonal antibody, APO2.7 was developed against the 38 kDa mitochondrial membrane protein 7A6 antigen. Since the 7A6 antigen is selectively expressed on the mitochondrial membrane in cells undergoing apoptosis, we attempted to detect apoptotic cells using this monoclonal antibody after 3% ethanol treatment of HL-60 cells. As shown in Fig. 6, APO2.7 antibody staining of unprocessed cells rapidly increased from 6% at 4h to 78% at 24h. However, following permeabilisa-



Figure 4. Kinetics of changes in cell size (forward scatter) after incubation of HL-60 cells with 3% ethanol.

Table 1. Percentages of apoptotic cells and of cells in other cell-cycle phases after incubation with 3% ethanol as determined by flow cytometry. Data are shown as mean values  $\pm$  SD of three independent experiments.

Time (h)	Apoptotic	G1	S	G2
$\begin{array}{c} 0 \\ 4 \end{array}$	$2.9 \pm 1.8 \\ 59.9 \pm 6.0$	$39.1 \pm 2.2$ $29.6 \pm 8.3$	$43.6 \pm 3.8 \\ 10.2 \pm 8.7$	$\begin{array}{c} 14.3 \pm 2.3 \\ 0.3 \pm 0.4 \end{array}$
$\frac{7}{24}$	$59.5 \pm 8.0 \\ 74.8 \pm 0.5$	$32.8 \pm 6.8$ $24.8 \pm 0.4$	$\begin{array}{c} 7.4 \pm 9.5 \\ 0.0 \pm 0.0 \end{array}$	$\begin{array}{c} 0.3 \pm 0.3 \\ 0.4 \pm 0.1 \end{array}$

tion of ethanol-treated HL-60 cells APO2.7 antibody could be seen to identify 91% of cells as apoptotic as early as after 4h (Fig. 7).

Correlation between cell size and APO2.7

16 and 24 h after addition of 3% ethanol, cell size (FS) was correlated with APO2.7 antibody staining of unprocessed cells. Cells with high FS were not stained by



Figure 5. Flow cytometric analysis of DNA content and cell-cycle after 4 hours of incubation of HL-60 cells with 3% ethanol. Apoptotic cells were identified as cells with subdiploid DNA content (lower DNA content than cells in  $G_0/G_1$  phase), i.e.  $subG_1$ peak. Representative results for single normal control (left) and single experiment (right) are shown.

**Table 2.** Percentages of apoptotic cells determined by different methods after incubation with 3% ethanol. Data are shown as mean values  $\pm$  SD of three independent experiments.

		Time of incubation (h)				
Method	0	4	7	24		
morphology subG <sub>1</sub> peak APO2.7 unprocessed cells APO2.7 permeabilised cells	$\begin{array}{c} 2.8 \pm 2.2 \\ 5.4 \pm 2.7 \\ 2.7 \pm 2.2 \\ 10.6 \pm 6.2 \end{array}$	$\begin{array}{c} 55.4 \pm 9.8 \\ 59.4 \pm 7.4 \\ 6.1 \pm 2.7 \\ 91.1 \pm 7.9 \end{array}$	$\begin{array}{c} 93.3 \pm 3.2 \\ 59.5 \pm 9.8 \\ 8.1 \pm 4.0 \\ 96.5 \pm 2.1 \end{array}$	$\begin{array}{l} 99.0\pm1.0\\ 75.4\pm1.1\\ 78.1\pm11.5\\ 95.1\pm1.4\end{array}$		

APO2.7 and can be considered as live cells. After 16 hours of incubation with 3% ethanol, 50% of small cells with low FS were APO2.7 positive, and 24 hours after ethanol addition 63% of these cells were APO2.7 positive (Fig. 8).

# Comparison of different methods of apoptosis detection

After 4 hours morphologic changes were observed in the nuclear and cytoplasmic structures using Diff-Quik stained cytospin preparations and standard light microscopic techniques (50% apoptotic cells). Comparable results were obtained by flow cytometric measurement of sub-diploid DNA content (sub-G<sub>1</sub> cells). After staining with APO2.7 antibody in cells permeabilised by digitonin 91% of the cells showed positivity after 4 hours. After 7 hours almost all cells exhibited apoptotic mor-







**Figure 8.** Correlation between cell size and APO2.7-PE fluorescence intensity of unprocessed HL-60 cells after apoptosis inducted by 3% ethanol. Cells with high FS (region E) were not stained by APO2.7 (top) and could be considered as live cells. Small cells with low FS were stained by APO2.7 (bottom) to 50% (16h) and 63% (24h) and were considered apoptotic.

phology. Staining of unprocessed cells with APO2.7 seems to be a later indicator, positivity increasing from 8% after 4 hours to 78% after 24 hours (Tab. 2).

#### Discussion

Incubation of human myeloid leukemia cells with 10% ethanol leads to their death by necrosis (Kravtsov and Fabian 1996). Similarly as  $H_2O_2$  ethanol is among the stimuli after which cells die by apoptosis or necrosis in a dose-dependent manner. In our work we could prove that during 48 hours of incubation with 1–4% ethanol HL-60 cells die by apoptosis. In accordance with the results of Aroor and Baker (1997) we did not observe any induction of apoptosis after treatment with 0.5% ethanol in this time interval. However, if the incubation with 0.5% ethanol is prolonged till 4 days, even this concentration induces apoptosis in 25% of cells and decreases proliferation (Aroor and Baker 1997).

One of the methods used for determination of apoptosis should be a morphologic method. Our results show that incubation of HL-60 cells in culture medium with 2 and 3% ethanol induces apoptosis in a dose-dependent manner as early as after 4 hours. Diff-Quik stained cytospin preparations were analyzed using standard light microscopy at  $1000 \times$  magnification. Striking apoptotic cellular changes observed by microscopy include cell shrinkage of up to 30%, crescent-shaped masses of condensed chromatin adjacent to the nuclear envelope, membrane blebbing, and cytoplasmic and nuclear fragmentation leading to the formation of apoptotic bodies (Allen et al. 1997). During apoptosis DNA becomes fragmented by endonucleases and these small DNA fragments can break out from the cells, resulting in a reduced total DNA content and hence a sub- $G_1$  fluorescence peak representing apoptotic cells. HL-60 cells treated with ethanol show an increase in the sub- $G_1$  fraction upon cytometric analysis of DNA content by PI staining. Using this method we obtained results corresponding with the morphologic method. An apoptotic peak was observed 4 hours after addition of 2% and 3% ethanol, and after 48 hours after 3% ethanol addition only sub-G<sub>1</sub> cells were observed. The staining with APO2.7 in cells permeabilised by digitonin was shown as a likewise early indicator of apoptosis. A decrease of cell size as determined by FS values was apparent mainly in later intervals, after 16–24 hours, while changes observed until 7 hours after the beginning of incubation were insignificant. A similar marker is APO2.7 staining in nonpermeabilised cells, which is apparent from 16 hours on after the beginning of incubation, and is related to cell membrane lesion and the possibility to determine APO2.7 on mitochondrial membrane. The results showed that after 16 and 24 hours after the beginning of incubation with 3% ethanol the cells with high FS are APO2.7 negative, while 50 and 63% (after 16 and 24 hours respectively) of the cells with low FS are APO2.7 positive. CD95 antigen expression is low in HL-60 human promyelocytic cell-line (2%) and is not affected by ethanol-induced apoptosis. Upon CD95 antibody inducted apoptosis, unprocessed cells stained with anti-tubulin-FITC (Koester et al. 1997). They also showed similar responses of Trypan blue and APO2.7-PECy5, with the most significant increase in percentages of positive cells occurring between 6 h (15-18%) and 12 h (47-53%) after induction. Light-scatter changes started to appear at 4.5 h (15%) and increased slightly faster than anti-tubulin antibody, Trypan blue and APO2.7 antibody staining in unprocessed cells. Annexin V values from untreated cells started higher at time 0 h (17%) compared with all other methods (3–6%) analyzed by Koester et al. (1997), however starting at 3 h APO2.7-PECy5 in processed digitonin-permeabilised cells and DNA strand break values rose more rapidly than with Annexin V, but all the three probes showed similar responses (87–91%) by 12 h.

The antigen identified by APO2.7 appears relatively early in the apoptotic process and thus, when the cell has become permeable as a result of events in late apoptosis/necrosis it is no longer necessary for the investigator to use a permeabilisation agent, since the antigen is available for staining (Koester et al. 1997).

Our results with apoptosis induced in human promyelocytic leukemia cells HL-60 using 3% ethanol showed that with respect to early apoptosis (4h) the results of APO2.7 staining in permeabilised cells, morphological determination of apoptosis using Diff-Quik stained cytospin preparations and sub-G<sub>1</sub> fraction after PI staining of DNA are all comparable. However, the decrease of cell size identified by FS and APO2.7 staining in nonpermeabilised cells are signs of late apoptosis.

Acknowledgements. This work was supported by grants from the Czech Ministry of Defense (MO66020398129) and from Grant Agency of the Charles University (GA UK 279/98).

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Final version accepted March 16, 2000