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Resistance of Mitochondrial DNA to Degradation Characterizes the Apoptotic but Not the Necrotic Mode of Human Leukemia Cell Death

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Abstract Cell death can occur by two basically different processes. The original term, *necrosis,* is now reserved for the generally destructive series of events which include the release of lysosomal enzymes and loss of cell membrane integrity. In contrast, mild treatment with cell damaging agents, or withdrawal of growth factors, may result in a characteristic form of degradation of cellular DNA which is associated with cell death that has morphology known as apoptosis. In this study human leukemia cells were exposed to agents or conditions previously reported to cause necrosis or apoptosis, monitored by detection of DNA "ladders," and the integrity of cellular DNA was determined on Southern blots. Nuclear DNA was distinguished from mitochondrial DNA by use of probes specific for nuclear genes or for mitochondrial DNA. When HL60, K562, MOLT4, or U937 cells were exposed to conditions which resulted in necrosis, mitochondrial DNA was damaged at approximately the same rate as nuclear DNA, but in apoptosis mtDNA was not degraded. Thus, the ratio of the relative (to untreated cells) abundance of mitochondrial DNA measured by a probe for 16S mitochondrial ribosomal RNA on Southern blots, to the relative abundance of DNA of any nuclear gene, was 1 or less in necrosis, but rose to values greater than 2 in apoptosis. It is concluded that the comparison of the degree of fragmentation of mitochondrial and nuclear DNA provides a quantitative way of distinguishing necrosis from apoptosis.

Key words: necrosis, cell death, cell membrane integrity, lysosomal enzymes, apoptosis

It is becoming well established that cell death is one of the important mechanisms which control an excessive expansion of cell populations and the morphogenesis of a developing organism. It therefore follows that there must be cellular programs which lead to the death of cells in certain situations, such as the unneeded cell masses in the embryo, cells desquamating from the epithelial surfaces, or the deletion of "forbidden" clones of T lymphocytes [Alison and Sarraf, 1992; Glucksmann, 1951; Smith et al., 1989]. This form of cell death has been characterized morphologically, both by light microscopic and electron microscopic studies, and has been termed apoptosis, to distinguish it from necrosis, which is thought to be a more passive degenerative phenomenon [Searl et al., 1982; Wyllie et al., 1980]. Apoptosis can also be recognized at the molecular level by the preferential cleavage of internucleosomal DNA, which results in the appearance of a series of bands when DNA extracted from apoptotic cells is size-fractionated on agarose gels [Wyllie et al., 1984]. These bands, commonly known as "DNA ladders," are multiples of 180 base pairs (bp) of nucleotides that form a nucleosome. The nucleosomal ladders provide an indication that nuclear DNA endonucleases have been activated, but few other biochemical correlates of apoptosis have been described. Yet it seems certain that additional objective criteria for classifying cell death are needed if advances in the study of this biological phenomenon are to be made.

Mitochondria and other cellular organelles are known to be preserved in apoptosis, but undergo rapid morphological degradation in necrosis [Searl et al., 1982; Wyllie et al., 1980]. Changes of necrosis are preceded by cellular edema that leads to swelling and rupture of cellular organelles, including the lysosomes, which release a variety of hydrolyzing enzymes. In contrast,

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apoptosis is characterized by nuclear condensation and cytoplasmic shrinkage, accompanied by a release of apoptotic bodies, which represent membrane-bound cellular organelles being extruded from the cell. Intact mitochondria are frequently seen in such apoptotic bodies [Kerr et al., 1972].

An interesting link between mitochondria and apoptosis is provided by the recent observations that the product of the nuclear gene bcl-2 localizes to the inner mitochondrial membrane, and that its overexpression inhibits apoptosis [Hockenbery et al., 1990; Sentman et al., 1991; Vaux et al., 1988]. Mitochondria contain a number of copies of a 16.5 kbp circular DNA genome which encodes a portion of mitochondrial proteins and the mitochondrial ribosomal and transfer RNAs [Anderson et al., 1981; Clayton, 1984]. The stability of this DNA exposed to agents and conditions which damage nuclear DNA (nuDNA) has been studied in mouse thymocytes and P-815 mastocytoma cells [Murgia et al., 1992], but mtDNA stability in human cells exposed to agents which cause apoptosis or necrosis has not been compared.

We have recently found that the topoisomerase II inhibitor teniposide, also known as VM-26, has differential effects on the integrity of DNA in human leukemia cells [Tepper and Studzinski, 1992]. The degradation of nuDNA was characterized by the appearance of nucleosomal "ladders" and other features which indicate the apoptotic mode of cell death [Kerr et al., 1972; Wyllie, 1980], but mitochondrial DNA (mtDNA) was relatively intact. It was important to determine if mtDNA is spared during the death of leukemic cells in general, if it is a characteristic of apoptosis, or if this is a specific feature of the mode of action of teniposide.

MATERIALS AND METHODS

Cell Lines and Culture

HL60 human promyelocytic leukemia, K562 chronic myelogenous leukemia in blast crisis, MOLT-4 acute lymphoblastic leukemia, and U937 histiocytic lymphoma (with characteristics of monocytic leukemia) cell lines were grown in suspension in RPMI 1640 medium (Mediatech, Washington, DC), supplemented with 10% fetal calf serum (Hycone Laboratories, Inc., Logan, UT), 2 mM glutamine (Mediatech), and 50 I.U./ml penicillin-50 μg/ml streptomycin (Mediatech). The HL60 cells used were a well-differentiating subclone, HL60-G1 [Kolla et al., 1991], and a 1-β-D-arabinofuranosylcytosine (Ara-C) resistant clone HL60-AraC [Bhalla et al., 1984]. K562, MOLT-4, and U937 cells were obtained from ATCC (Rockville, MD). All cultures were incubated at 37°C in a humidified environment of 5% CO_2/95% air. All cell lines were confirmed to be free of mycoplasma infection by an autoradiographic method [Studzinski et al., 1985]. Cell numbers were determined using a Neubauer hemocytometer.

Chemicals

Ara-C (Sigma, St. Louis, MO) and doxorubicin-hydrochloride (Sigma) were prepared as 10 mM stock solutions in Milli-Q H_2O and stored at −20°C until use. Stock solutions of 5 mg/ml VP-16 (etoposide), 10 mM VM-26 (teniposide, a generous gift of Bristol-Meyers), and 5 mM calcium ionophore A23187 (Sigma) were prepared in DMSO and stored at −20°C. All solutions were sterilized by filtration through 0.2-μm Millipore filters.

Determination of Mitochondrial Respiratory Function and Cell Membrane Integrity

Mitochondrial respiratory activity was assessed by monitoring the ability of cells to reduce the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to its formazan [Mossman, 1983]. Ten microliters of a sterile-filtered 5 mg/ml solution of MTT in phosphate-buffered saline was added to 100 μl of cells in 96-well plates and incubated at 37°C for 2.5 h. Cells with functioning mitochondria contained blue crystals and could easily be enumerated with a Neubauer hemocytometer. Cell membrane integrity was determined by the ability of the cells to exclude the vital stain trypan blue (TB), also counted in a hemocytometer.

DNA Isolation

DNA was isolated from cell cultures using slight modifications of established methods [Enrietto et al., 1983; Gross-Bellard et al., 1972]. Cells were lysed with a digestion buffer containing 100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% SDS, and 0.2 mg/ml proteinase K, and incubated at 50°C for 12 h. Lysates were then extracted once with phenol/chloroform/isoamyl alcohol (25:24:1), and twice with chloroform/isoamyl alcohol (24:1) followed by precipitation with 2.5 M ammonium acetate

and 100% ethanol. The DNA was collected by centrifugation at 4,000 rpm for 30 min in a Beckman J-6 centrifuge at room temperature. The pellets were washed with 70% ethanol, and centrifugation repeated. The DNA was then air-dried and dissolved in TE buffer (10 mM Tris-Cl, 5 mM EDTA, pH 8.0). Aliquots were taken for spectrophotometric readings at 260 nm and 280 nm to determine both the concentration and quality of the DNA [Maniatis et al., 1982]. This procedure always yielded a 260/280 value of 1.8 or higher.

Analysis of DNA Fragmentation

Eight micrograms of each DNA sample was incubated with 0.1 units of DNase-free RNase (BMB, Indianapolis, IN) for 1 h at 37°C, and the samples were loaded onto a 2% agarose gel. Electrophoresis was carried out in TAE buffer (10 mM Tris-acetate, 1 mM EDTA, pH 8.5) at 4 V/cm for 7 h. The gel was stained with ethidium bromide (0.5 μg/ml in distilled water), destained for approximately 2 h in several changes of water, and then photographed with Polaroid 665 positive/negative film by UV illumination.

Southern Blot Analysis

One hundred micrograms of each DNA sample to be analyzed was digested with the restriction enzyme Eco RI or Hind III (3.5 units/μg DNA) for 8 h at 37°C. This was followed by treatment of each reaction with 2 units of DNase-free RNase for 1 h at 37°C. All reactions were terminated by organic extractions as described above under DNA Isolation. The digested DNA samples were precipitated with 0.2 M NaCl and 100% ethanol and ultimately dissolved in TE (pH 8.0) and quantitated. Ten micrograms of each DNA sample was electrophoresed on a 1.2% agarose gel in TAE buffer for 16 h at 1 V/cm followed by depurination and denaturation as described by [Wahl et al., 1979]. The DNA was then transferred to Biotrans nylon membranes (ICN) in 20× SSC for 16–24 h as described by Southern [Southern, 1975]. The DNA was immobilized on the membrane by air-drying and subsequent baking at 80°C for 2–5 h. Prehybridization of the membranes was conducted at 42°C water bath in a buffer containing 50% formamide, 5× SSC, 5× Denhardt's solution, 200 μg/ml denatured and sheared salmon sperm DNA, and 0.5% SDS. Probes were nick-translated [Maniatis, 1982] and added to the hybridization buffer (1.2×10^6 cpm/ml) for 24 h. The membranes were then consecutively washed in 2×, 0.5×, and 0.1× SSC buffers containing 0.1% SDS at room temperature for 15 min per wash, followed by a final wash with 0.1× SSC/0.1% SDS at 42°C for 30 min. The membranes were then wrapped in plastic and exposed to autoradiographic film at −80°C for variable periods of time.

DNA Probes

The mitochondrial DNA (mtDNA) probe, p72, is a 600-bp Mbo I fragment of mtDNA from A-549 human lung adenocarcinoma cells inserted into the Bgl II site of the pML-TK-Bgl II vector [Tepper et al., 1992]. The probe for the human immunoglobulin λ light chain constant region (C_λ) gene was a 3.5 kbp genomic fragment inserted into pBR322. The human bcl-2 proto-oncogene probe, obtained from Oncor (Gaithersburg, MD), was a purified 2.8-kb Eco RI-Hind III fragment from the major breakpoint region of the gene. A 1.4-kb Cla I-Eco RI genomic fragment containing the third exon of the human c-myc proto-oncogene was used as a probe for that gene (Oncor).

RESULTS

Comparison of the Integrity of Mitochondrial and Nuclear DNA During Apoptosis and Necrosis

In order to establish the mode of cell death as a baseline for further experiments, HL60-AraC cells were exposed to a variety of conditions that were previously reported to produce either apoptosis or necrosis [Lennon et al., 1991]. With the exception of exposure to Ara-C to which these cells are resistant, all treatments to which the cells were subjected resulted in cell death as judged by a marked reduction of the conventional indices of viability, i.e., trypan blue exclusion and the MTT reaction (Table I). Necrosis-inducing conditions used in these experiments, DMSO (30%) and hyperthermia (50°C), produced a rapid loss of mitochondrial function (the MTT reaction) in virtually all cells. To demonstrate apoptosis the DNA extracted from these cells was subjected to 2% agarose gel electrophoresis and visualized with UV light after ethidium bromide staining (Fig. 1). Under these conditions high molecular weight DNA remains within the agarose gel wells, or enters the gel and migrates slower than the 23 kbp molecular weight marker. Degraded DNA enters the gel,

TABLE I. Indices of Viability and Relative mtDNA Integrity in HL60-AraC Cells Exposed to Conditions Inducing Apoptosis or Necrosis

Group[a]	Treatment	Prominence of DNA "ladders"	Percentage viability: Trypan blue exclusion	Percentage viability: MTT reaction	$p72/C_\lambda$ ratio[b]	Type of cell death
1	Control (untreated)	–	85.0 ± 1.0	74.7 ± 2.7	1.00	—
2	Ara-C (10 μM, 24 h)	–	75.0 ± 8.0	78.0 ± 2.0	1.19 ± 0.45	Resistant
3	Teniposide (5 μM, 8 h)	++	42.0 ± 3.0	26.3 ± 2.3	7.47 ± 2.65	Apoptosis
4	A23187 (5 μM, 24 h)	+++	13.3 ± 2.3	0.7 ± 0.7	114.01 ± 66.94	Apoptosis
5	DMSO (10%, 6 h)	+/–	46.0 ± 9.0	15.3 ± 6.3	2.78 ± 2.20	Apoptosis
6	DMSO (30%, 1 h)	–	8.7 ± 2.7	0	0.41 ± 0.09	Necrosis
7	Heat (44°C, 3 h)[c]	–	65.0 ± 9.0	27.0 ± 3.0	1.78 ± 0.48	?
8	Heat (50°C, 1 h)[d]	–	38.0 ± 2.0	0	0.56 ± 0.10	Necrosis

[a]The Southern blots for these groups are illustrated in Figure 2.
[b]Nuclear DNA and mtDNA levels were compared directly by sequential hybridization of the same membrane with probes for several representative nuclear genes, and a probe for mtDNA. Following densitometry of the autoradiographs, the ratios of the relative values of mtDNA/nuclear gene (e.g., bcl-2, C_λ) were obtained for untreated cells ("controls") and for each treated group. Control mtDNA/nuDNA ratios were converted to a value of 1.00, and the "treated" mtDNA/nuDNA ratios were multiplied by the same conversion factor yielding the values presented in Tables I–IV. The values represent means ± S.E.M. of three determinations.
[c]Incubated for 6 h at 37°C following heat treatment.
[d]Incubated for 1 h at 37°C following heat treatment.

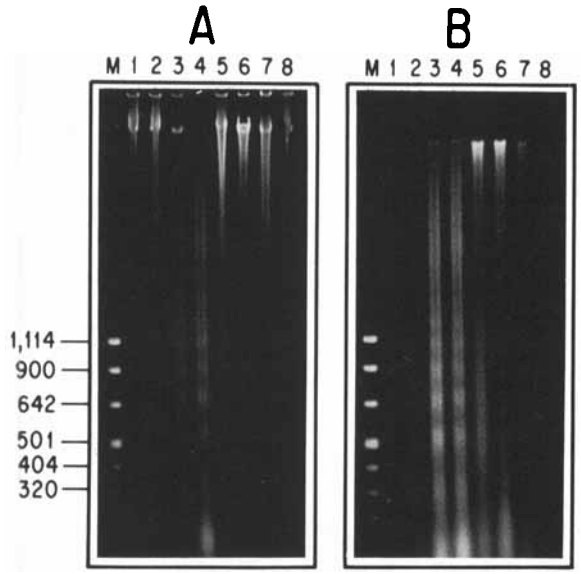

Fig. 1. Comparison of the patterns of DNA degradation during conditions inducing apoptosis or necrosis in HL60-AraC cells. Following the treatments indicated below, the isolated DNA was precipitated at room temperature **(A)** and subjected to 2.0% agarose gel electrophoresis. In addition, DNA remaining in the supernatants was precipitated at −20°C and analyzed similarly **(B)**. Samples were arranged on the gels as follows: **lane 1,** untreated control; **lane 2,** Ara-C (10 μM); **lane 3,** VM-26 (5 μM); **lane 4,** calcium ionophore A23187 (5 μM); **lane 5,** DMSO (10%); **lane 6,** DMSO (30%); **lane 7,** heat (44°C); **lane 8,** heat (50°C). A restriction enzyme digest (Hpa I and Dra I + Hind III) of pUCBM21 DNA served as a molecular weight marker **(M)**. Durations of all treatments are indicated in Table I. In panel B, groups 1, 2, and 8 contained no low molecular DNA.

and internucleosomal endonuclease activity results in the appearance of "ladders" comprised of DNA of 180 bp multiples protected by core histones [Hewish and Burgoyne, 1973]. The DNA was examined both in samples precipitated by ethanol at room temperature (Fig. 1A), and in samples of the supernatant fluid from this precipitation which primarily contained DNA of low molecular weight (Fig. 1B). Exposure to teniposide (5 μM) or calcium ionophore A23187 (5 μM) resulted in degradation of internucleosomal DNA, indicative of apoptosis (Fig. 1A,B, lanes 3 and 4). Exposure to 10% DMSO resulted in faint nucleosomal ladders, which were clearly recognizable by UV examination of ethidium bromide-stained gels, but which did not photograph well (Fig. 1A,B, lanes 5). In contrast, nucleosomal ladders could be detected in cells treated with 30% DMSO, or exposed to 50°C (Fig. 1A, lanes 6 and 8). MTT and TB tests of viability showed that the cells were dead by these conventional criteria thus indicating that these cells underwent necrosis (Table I).

Enumeration of total cell numbers showed that there was no loss of cells from the cultures (data not shown), showing that in the time frame of these experiments cell disintegration did not take place to an appreciable extent.

Comparison of DNA integrity in nuclei and mitochondria was performed by Southern blot analysis of the DNA samples shown in Figure 1. Subsequent quantitation of the relative band

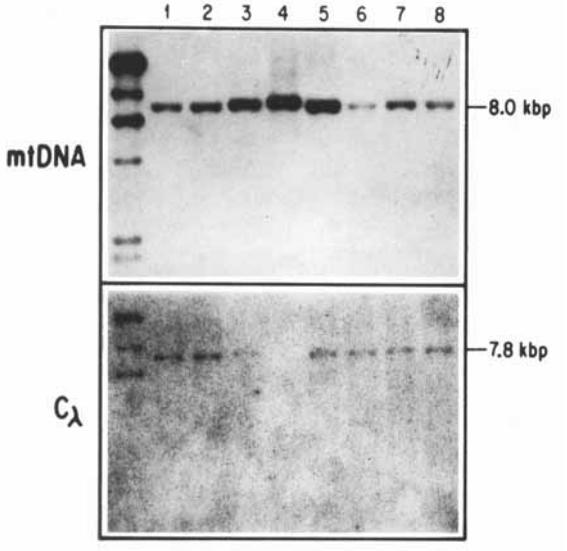

Fig. 2. Mitochondrial and nuclear DNA levels following apoptosis or necrosis DNA samples from the experiment shown in Figure 1A were digested with Eco RI and subjected to Southern blot analysis mtDNA was examined with the p72 probe **(top)**. The membrane was stripped and re-hybridized with a probe for the immunoglobulin λ light chain constant region (C_λ) gene, a 3 5-kbp genomic fragment inserted into pBR322 as a representative nuclear gene **(bottom)**. The first lane of the autoradiogram contains Hind III-digested bacteriophage λ DNA molecular weight markers

intensities of mtDNA, detected by the p72 probe which contains a portion of the 16S mitochondrial ribosomal RNA gene [Tepper et al., 1992], and the immunoglobulin lambda light chain constant region (C_λ) gene, a representative nuclear gene, showed that conditions which cause necrosis reduced the p72/C_λ ratio by approximately a factor of two. Repetition of this experiment with probes for several other nuclear genes (eg, c-myc, c-Ha-ras, 18S rRNA) gave essentially the same results (data not shown). In contrast, conditions which produced nucleosomal ladder generation (i.e., apoptosis) caused a greater than two-fold increase in the mtDNA/nuclear gene DNA ratio, and this increase was proportional to the apparent intensity of nucleosomal ladders on agarose gels of precipitated DNA (Figs. 1A, 2, and Table I). For example, (a) A23187 produced very prominent agarose gel ladders (Fig. 1A,B, lane 4) and a very high mitochondrial DNA (p72) to nuclear DNA (C_λ) ratio (Fig. 2, lane 4); (b) 10% DMSO produced faint DNA ladders (Fig. 1A,B, lane 5) and a low p72/C_λ DNA ratio (Fig. 2, lane 5); and (c) VM-26 (Figs. 1A,B, 2, lanes 3) was intermediate in both respects. Although it was reported that heating HL60 cells at 44°C for 3 h results in apoptosis [Lennon et al., 1991], such treatment of the variant of HL60 cells used in these experiments (HL60-AraC) did not produce visibly detectable DNA "ladders" (Fig. 1A,B, lanes 7), and the mtDNA/C_λ ratio was only slightly increased (Fig. 2, Table I). Thus, the biochemical correlates of apoptosis were not evident in these cells following heat treatment. These experiments show that in HL60-AraC cells mitochondrial DNA is spared in apoptosis but not in necrosis, and that a quantitative assessment of the extent of apoptosis can be obtained.

Effects of Doxorubicin on Mitochondrial DNA During Apoptosis and Necrosis

A lack of penetration of the mitochondrial membrane by apoptosis-inducing chemical agents which interact with DNA could be a factor in a failure to produce mtDNA damage. This was tested directly using the cancer chemotherapeutic drug doxorubicin (DOX), which is known to localize to the inner mitochondrial membrane, and to form complexes with cardiolipin [Goormaghtigh et al., 1980]. As is shown in Figures 3 and 4, DOX treatment of HL60-G1 cells resulted in the production of nucleosomal ladders and degradation of C_λ DNA, but mitochondrial DNA was remarkably intact relative to nuclear DNA. In contrast, in K562 cells treated with DOX did not produce nucleosomal DNA ladders. Loss of viability of K562 cells observed at 24 h was similar to the loss of viability in HL60 cells at 12 h of treatment, in which clearly visible nucleosomal ladders were demonstrated (Table II, Fig. 3). Interestingly, in K562 cells treated with DOX there was loss of mtDNA, and this loss was detectable on Southern blots earlier (at 12 h in Fig. 4) than the loss of viability measured by either TB exclusion or by the MTT method (noted only at 24 h, Table II). Although a part of the decreased intensity of mtDNA bands could be due to a block to DNA replication, the magnitude and rapidity of this decrease indicated that damage to mtDNA was also taking place. Thus, the features of K562 cell death produced by DOX indicated necrosis, and mtDNA was damaged, as shown in experiments in which necrosis was induced by 30% DMSO or by exposure to hyperthermia at 50°C.

Mitochondrial Function and DNA Integrity

In order to compare the time course of changes in several parameters that indicate cell death,

Table II. Comparison of the Rates of Loss of Viability and Relative mtDNA Integrity in HL60-G1 and K562 Cells Exposed to Doxorubicin

	HL60-G1 cells[a]			K562 cells[b]		
Duration of exposure (h)	Trypan blue exclusion[c]	MTT reaction[d]	mtDNA/C_λ ratio[e]	Trypan blue exclusion[e]	MTT reaction[d]	mtDNA/C_λ ratio[e]
0	90 ± 3.7	86 ± 2 3	1.00	98 ± 1.0	99 ± 3.0	1.00
2	93 ± 1.3	86 ± 0 7	0.93 ± 0.01	99 ± 1.0	98 ± 1 3	0.64 ± 0.03
4	88 ± 6.3	92 ± 2 0	0.90 ± 0.05	98 ± 0.7	93 ± 4.3	0.61 ± 0.05
8	87 ± 2.0	68 ± 1 7	1.12 ± 0.05	98 ± 1.3	95 ± 1.3	0.57 ± 0.04
12	74 ± 1.0	60 ± 2 7	2.36 ± 0.04	95 ± 2.7	91 ± 1.3	0.22 ± 0.03
24	44 ± 3.0	7 ± 5 0	4.83 ± 0.45	71 ± 0.7	61 ± 3.0	0.25 ± 0.06

[a]Treated with 5 μM doxorubicin
[b]Treated with 10 μM doxorubicin
[c]Percentage of cells excluding trypan blue
[d]Percentage of cells positive for the MTT reaction
[e]Defined in footnote b to Table I

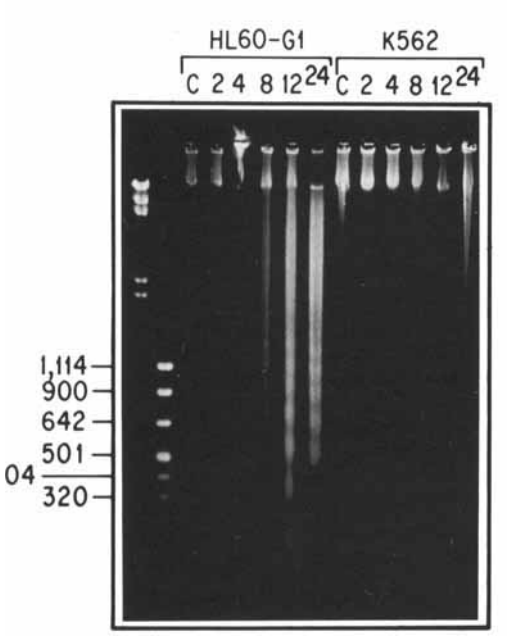

Fig. 3. Doxorubicin treatment produces nucleosomal ladders in HL60-G1 cells, but not in K562 cells HL60 cells and K562 cells were exposed to 5 μM and 10 μM DOX, respectively, followed by 2 0% agarose gel electrophoresis Duration (in hours) of exposure to the drug appears above each lane DNA from control cell cultures (C) was also isolated at the 24 h time point The first two lanes contain Hind III-digested bacteriophage λ DNA, and pUCBM21, as molecular weight markers

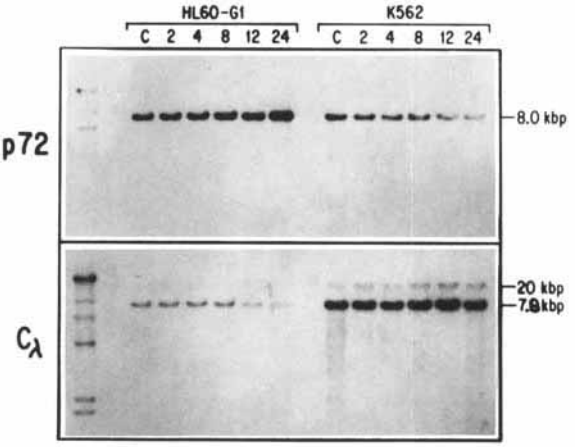

Fig. 4. Southern blot analysis of Eco RI-digested DNA from experiment shown in Figure 3 The same nylon membrane was probed successively with probes for mtDNA and C_λ

HL60-G1 cells were exposed to teniposide for periods of up to 12 h. This resulted in a rapid decline in mitochondrial function, determined by monitoring the ability of cells to reduce the tetrazolium salt MTT [Mosmann, 1983], and was followed by a loss of cell membrane integrity, shown by the inability of the cells to exclude trypan blue (Fig. 5). DNA isolated from these cells was examined by gel electrophoresis. Only high molecular weight DNA was observed during the first 3 h of teniposide exposure, but after this time evidence of internucleosomal endonucleolytic activity was observed, as shown by the generation of nucleosomal "ladders" (Fig. 6). During this widespread damage to nuDNA, degradation of mtDNA was not detected (Fig. 7, upper panel), as we previously reported [Tepper and Studzinski, 1992]. In contrast, the genomic DNA, as illustrated by the bcl-2 proto-oncogene DNA, which encodes an apoptosis-inhibitory protein that localizes in the mitochondria [Hockenbery et al., 1990; Sentman et al., 1991; Vaux et al., 1988], was degraded to approximately 30% of the DNA content of this gene in untreated cells (Fig. 7, lower panel). Similar results were obtained when any other probe for a nuclear gene was used [Tepper and Studzinski, 1992].

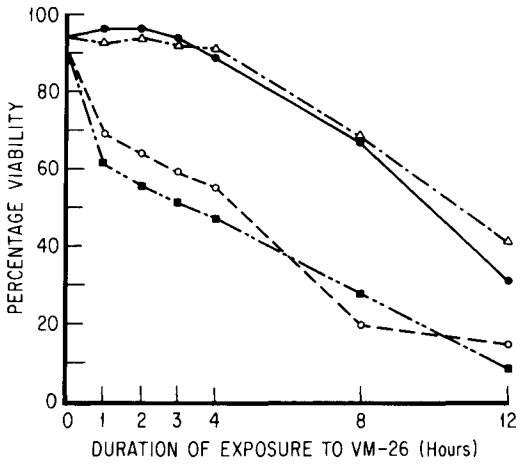

Fig. 5. Continuous exposure of HL60 promyelocytic leukemia cells to 5 μM teniposide (VM-26) results in a rapid loss of viability, as determined by monitoring mitochondrial function (○—○; ■—■) and cell membrane permeability (●—●; △—△), using the MTT and trypan blue exclusion assays, respectively. Data from two separate experiments are shown. Viability was monitored on a cell-to-cell basis by enumeration with a hemocytometer of the cells yielding a positive MTT reaction and cells excluding trypan blue.

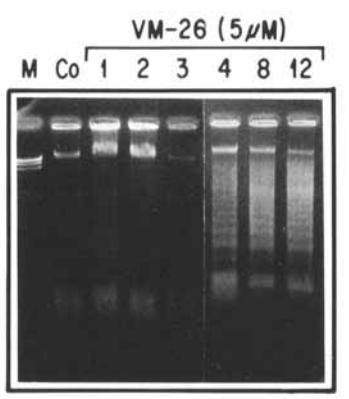

Fig. 6. Activation of internucleosomal endonuclease activity during VM-26 exposure. DNA was isolated from HL60 cells exposed to 5 μM VM-26 for up to 12 h, followed by 2.0% agarose gel electrophoresis. **Co** is untreated control DNA. Hind III-digested bacteriophage λ DNA was used as a molecular weight marker (**M**).

Following normalization of the values obtained from densitometric scanning of the Southern blots, the ratios of p72/bcl-2 band intensities were determined and were considered to indicate the integrity of mtDNA relative to the integrity of nuDNA (Table III). A marked increase in this ratio appeared after 3 h of VM-26 exposure, which coincided with the appearance of DNA "ladders" on ethidium bromide-stained agarose gels (Fig. 6). Thus, the bcl-2 gene is similar to

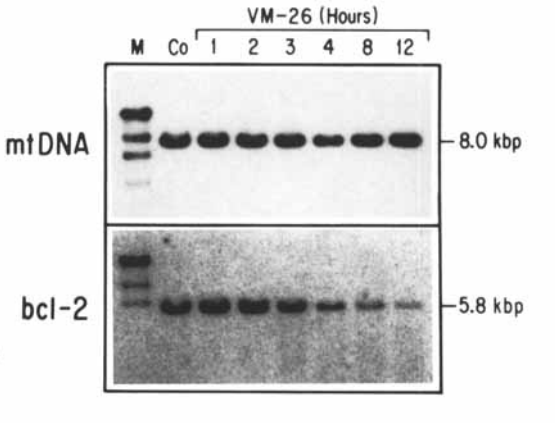

Fig. 7. Maintenance of mtDNA integrity while nuclear DNA is degraded. DNA samples (10 μg) from the experiment shown in Figure 6 were digested with Eco RI, and subjected to Southern blot analysis. The membrane was first hybridized with nick-translated plasmid p72, a probe for mtDNA **(top)**, followed by stripping and rehybridization with a probe for bcl-2 proto-oncogene, a purified 2.8-kb Eco RI-Hind III fragment from the major break point region of this gene (Oncor, Gaithersburg, MD) **(bottom)**. Approximate molecular weights were indicated by the mobility of bacteriophage λ DNA (Hind III digest) **(M)**.

TABLE III. mtDNA/bcl-2 Signal Intensity Ratios in HL60-G1 Cells Exposed to Teniposide

Duration of VM-26 exposure (h)	mtDNA/bcl-2 ratio[a]
0	1.00
1	0.89 ± 0.04
2	2.44 ± 0.71
3	7.05 ± 0.71
4	11.93 ± 1.25
8	10.80 ± 1.45
12	8.17 ± 2.36

[a]Expressed as the mean ± S.E.M. of three determinations. Description of the calculation is presented in footnote b to Table I.

the other nuclear genes in its susceptibility to apoptotic degradation.

Resistance of mtDNA to Digestion During Apoptosis Induced in Other Human Leukemic Cell Lines

Results presented above have been obtained using two human leukemic cell lines—promyelocytic leukemia HL60 cells, and chronic myelogenous leukemia K562 cells. In order to show that the resistance of mtDNA to degradation is a more general characteristic of apoptosis in leukemic cells, T cell lymphocytic leukemia MOLT-4 cells, and monocytic leukemia U937 cells were also studied. Only faint nucleosomal ladders re-

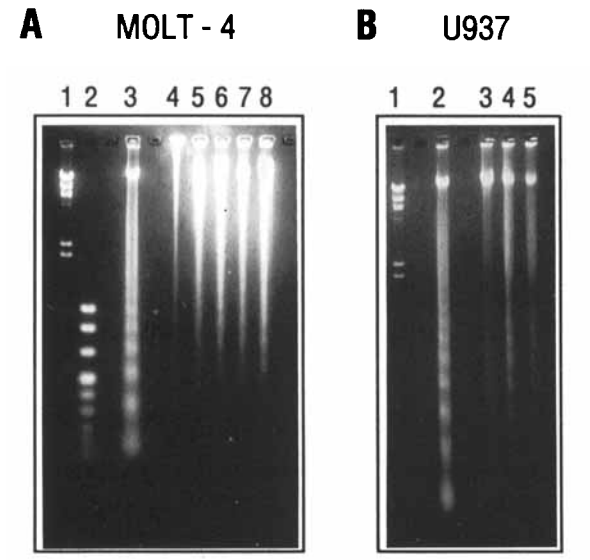

Fig. 8. DNA fragmentation during drug-induced apoptosis in MOLT-4 and U937 leukemia cells **A:** DNA was isolated from untreated MOLT-4 cells (**lane 4**), from cells exposed to 1 μM doxorubicin for 12 and 24 h (**lanes 5** and **6, respectively**), and from cells treated with 10 μM doxorubicin for 12 h (**lane 7**) and 24 h (**lane 8**). Following DNA isolation, samples were electrophoretically analyzed as described in the legend to Figure 1 **B:** Similar analysis of untreated U937 cells (**lane 3**), and cells exposed for 12 h to 5 μM VP-16 (**lane 4**), or 10 μM Ara-C (**lane 5**). DNA from HL60-G1 cells treated with the calcium ionophore A23187 (5 μM) served as a positive control for the presence of nucleosomal ladders (A, lane 3, B, **lane 2**) Molecular weight markers were Hind III-digested λ DNA (**lane 1**, A and B) and pUCBM21 (Hpa I, Dra I, and Hind III-digested) in **lane 2** of A only

sulted from treatment of MOLT-4 cells with doxorubicin (Fig. 8A), yet mtDNA was clearly resistant to degradation, as illustrated in Figure 9A. The more easily discernible nucleosomal ladders in U937 cells treated with VP-16 or Ara-C (Fig. 8B) were accompanied by the greater prominence of mtDNA bands on Southern blots, when compared to bands of genomic DNA (Fig. 9B). These experiments, quantitated as summarized in Table IV, again show that resistance of mtDNA relative to nuDNA is a sensitive indicator of the apoptotic mode of cell death.

DISCUSSION

Previous electron microscopical studies have shown that the morphological integrity of mitochondria tends to be preserved even when they are extruded from the cell in apoptotic bodies, but that in necrosis mitochondria usually have distorted architecture [Searl et al., 1982; Wyllie et al., 1980]. However, this does not show whether the entire population of mitochondria is preserved in apoptosis, or whether some mitochondria are destroyed, while other mitochondria are extruded intact from the dying cells. The results presented here, which show no loss of mtDNA while there is a loss of nuclear DNA and the appearance of nucleosomal ladders, provide evidence that the DNA of the bulk of the population of mitochondria is sheltered from drug-induced damage in apoptosis. This sug-

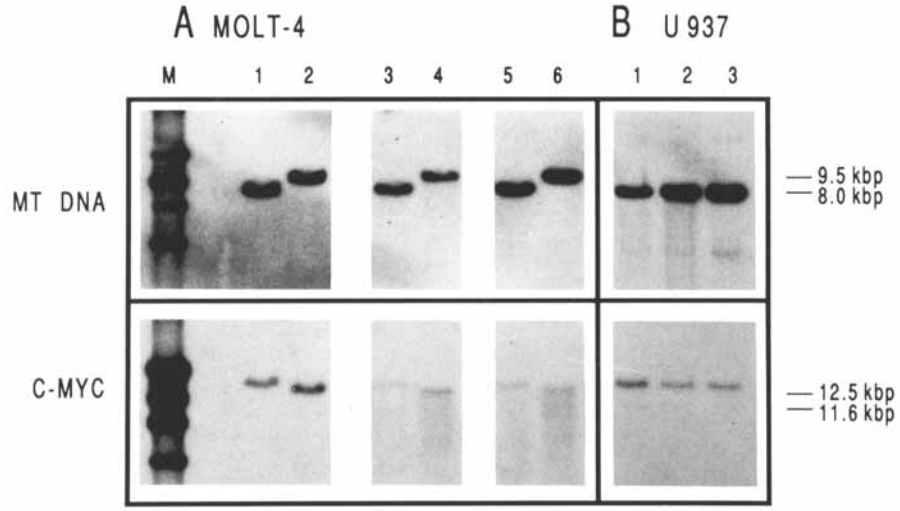

Fig. 9. Southern analysis of DNA from MOLT-4 and U937 leukemic cells exposed to chemotherapeutic agents Restriction enzyme digests of DNA samples shown in Figure 8 were subjected to Southern blot analysis with the p72 mtDNA and c-myc (exon 3) probes **A:** MOLT-4 DNA from untreated cells (**lanes 1** and **2**), or cells exposed to 10 μM doxorubicin for 12 h (**lanes 3** and **4**) and 24 h (**lanes 5** and **6**), was digested with either Eco RI (lanes 1, 3, and 5) or Hind III (lanes 2, 4, and 6) Bacteriophage λ DNA (Hind III-digested) was used as a molecular weight marker (**M**) **B:** Eco RI-digested DNA from untreated U937 cells (**lane 1**), and cells treated for 12 h with 5 μM VP-16 (**lane 2**) and 10 μM Ara-C (**lane 3**).

TABLE IV. Ratios of mtDNA to nuDNA During Apoptosis in MOLT-4 and U937 Leukemic Cell Lines

Cell line	Treatment	Concentration (μm)	Duration (h)	p72/c-myc ratio[a]	
				Eco RI	Hind III
MOLT-4	Control	—	—	1.00	1.00
	Doxorubicin	10	12	3.43 ± 0.56	2.50 ± 0 51
	DOX	10	24	7.50 ± 1.27	7 70 ± 0.92
U937	Control	—	—	1.00	
	Teniposide	5	12	4.18 ± 0 57	ND[b]
	ARA-C	10	12	4.27 ± 0.11	ND

[a]The ratio has been calculated as described in a footnote to Table 1
[b]DNA extracted from MOLT-4 cells was digested with Eco RI and Hind III, while DNA from U937 cells was digested with EcoR1 only

gests that activation of a nuclear, rather than a cytoplasmic, DNA endonuclease is responsible for the early events observable in apoptosis.

Recently Murgia et al. [1992] reported that mouse thymocytes and P-815 mastocytoma cells exposed to conditions which cause apoptosis showed the expected degradation of nuclear DNA, but mtDNA was not fragmented. These conditions included withdrawal of mitogenic factors and exposure to dexamethasone. Thus, apoptosis induced by means that mimic the conditions that may be encountered by the cells under physiological circumstances such as withdrawal of growth factors or interactions with hormones [Murgia et al., 1992], or resulting from exposure to chemotherapeutic agents [Tepper and Studzinski, 1992], shares similar characteristics.

The data in this report, along with results of other recent work [Tepper and Studzinski, 1992; Murgia et al., 1992], lead to the conclusion that one fundamental distinction between the two forms of death of leukemia cells is that mtDNA integrity is maintained during apoptosis, while mtDNA damage is an early feature of necrosis. If it is found to be true for other types of cells, this may prove to be an additional, highly sensitive, criterion for the distinction between apoptosis and necrosis.

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