

Apoptosis in Bone Marrow Biopsy Samples Involving Stromal and Hematopoietic Cells in 50 Patients With Myelodysplastic Syndromes

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Cell-cycle kinetics were measured *in situ* after infusions of iododeoxyuridine and/or bromodeoxyuridine in 50 patients with myelodysplastic syndromes (MDS) and the median labeling index in bone marrow (BM) biopsy samples was 28.6%. Unfortunately, 26 of 50 patients showed that $\geq 75\%$ of hematopoietic cells of all three lineages were undergoing programmed cell death (PCD) in their biopsy samples as shown by the *in situ* end labeling (ISEL) technique. Ten patients had 1/3 and eight had 2/3 ISEL⁺ cells. Stromal cells were frequently ISEL⁺ and often S-phase cells were also found to be simultaneously ISEL⁺. Nucleosomal DNA fragments as a ladder in agarose gel were present in BM aspirates

of four patients who showed high ISEL and were absent in two who had no ISEL staining in biopsy samples, but only when DNA was extracted after a 4-hour *in vitro* incubation in complete medium. Therefore, laddering data confirmed the ISEL findings that the majority of hematopoietic cells in MDS are in early stages of PCD. We conclude that extensive intramedullary cell death may explain the paradox of pancytopenia despite hypercellular marrows in MDS patients. Investigating approaches that protect against PCD in some MDS subsets would be of interest.

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THE MYELOYDYSPLASTIC syndromes (MDS) are a group of acquired hematopoietic disorders with evidence of trilineage dysplasia and an $\approx 30\%$ incidence of eventual transformation into acute myeloid leukemia (AML).¹⁻³ These are clonal disorders involving one or more clones,^{4,5} and normal hematopoiesis has been shown to simultaneously coexist in the majority of cases.⁶ Patients with evidence of "Abnormally Localized Immature Precursors" (ALIP) in their bone marrow (BM) biopsy samples tend to die earlier than ALIP-negative patients because of a rapid transformation into acute leukemia.⁷ Cytogenetic abnormalities especially involving chromosomes 5 and 7 are commonly detected,⁸ whereas ras-mutations signifying short survival have been showed in $\approx 10\%$ to 40% patients.⁹ The disease is almost invariably fatal, and with the exception of BM transplantation, there is no known cure.¹⁰

An apparent paradox in MDS is that patients with these disorders have peripheral cytopenias despite frequently having normo- or hypercellular BMs. One possible explanation for this contradictory finding may be that even though there are large numbers of cells in the BM, they are not exiting that compartment because they are undergoing premature programmed cell death (PCD). To examine this possibility, the present study was undertaken to define the incidence of PCD in the hematopoietic cells of MDS patients. Apoptosis is a gene-directed cellular self-destruction in which intracel-

lular endonucleases initially cleave the DNA into internucleosomal fragments (180 to 200 bp or their integral multiples).¹¹⁻¹⁴ This process, which begins with fragmentation of DNA, ends eventually with fragmentation of the nucleus and removal of the dead cell by macrophages. Although an end-stage karyorrhectic cell is easy to recognize under a light microscope, the earlier stages of PCD with limited DNA fragmentation and internucleosomal nicking cannot be morphologically identified. Thus, large numbers of cells may be undergoing apoptosis, yet there may be only a few cells in the morphologically identifiable karyorrhectic stage. Consequently, an accurate estimation of the incidence of PCD must include a precise quantitation of cells in early stages of apoptosis in addition to the obviously karyorrhectic cells. The conventional method of detecting typical "DNA laddering" by gel electrophoresis may not be able to provide this information because very little low molecular-weight DNA fragments are formed in cells that are in the earlier stages of PCD.¹⁵ The technique of *in situ* end labeling (ISEL) of fragmented DNA appears to be a more reliable measurement of the early stages of apoptosis.¹⁶⁻¹⁹ Therefore, BM biopsy samples of 50 MDS patients were processed for ISEL. The results obtained showed an exceptionally high rate of apoptosis in the hematopoietic cells of all three lineages in these patients. These data confirmed our initial hypothesis that high intramedullary death of hematopoietic cells may be the biologic basis for the paradox of pancytopenia despite hypercellular marrows in MDS patients.

MATERIALS AND METHODS

Rates of hematopoietic cellular proliferation and apoptosis were examined in fifty patients with a confirmed diagnosis of MDS. Each patient received a 1-hour infusion of bromo- and/or iododeoxyuridine (BrdU and IUdR) at 100 mg/m² as per protocols MDS 86-15 and/or MDS 90-02. Informed consent was obtained from every individual. The infusion protocols were approved by the local Institutional Review Board and the Food and Drug Administration and the drugs were provided by the National Cancer Institute. Some of these patients' results have also been reported in earlier studies.²⁰⁻²²

Studies of Cellular Proliferation

Immediately at the end of the second thymidine analogue infusion, samples of BM aspirate (BM asp) and biopsy were obtained. The aspirates were used for the determination of durations of S-phase as

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Submitted November 7, 1994; accepted February 9, 1995.

Supported in part by a Grant No. (R01CA60085) from The National Cancer Institute.

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0006-4971/95/8601-0026\$3.00/0

described before.^{23,24} The BM biopsy samples were embedded in plastic using glycol methacrylate. Air-dried 2-3- μm -thick sections were placed on Alcian Blue-coated coverslips and processed for the estimation of labeling indices using an anti-IUdR/BrdU monoclonal antibody (MoAb) as described before.^{23,24}

Studies to Measure the Incidence of PCD or Apoptosis

Two methods were used to measure the rate of apoptosis in these patients. The *in situ* end labeling (ISEL) of fragmented DNA as described by Wijsman et al¹⁶ was modified for use in plastic embedded biopsy samples for the detection of cells undergoing PCD. BM bpsps from six MDS patients, three AML patients, and two normal controls were examined for the presence of the typical ladder pattern in agarose gels. Details of both methods are provided below.

ISEL of Fragmented DNA

Two-micrometer sections from the previously mentioned BM biopsy samples were obtained. The sections were rehydrated in distilled water for 10 minutes and then incubated with freshly diluted 3% hydrogen peroxide (H_2O_2) for 30 minutes. Specimens were thoroughly rinsed in 0.15 mol/L phosphate buffer solution (PBS) (0.15 mol/L sodium chloride [NaCl] in 0.1 mol/L phosphate buffer, pH 7.5). Subsequently they were incubated with sodium chloride, sodium citrate solution (SSC) (0.3 mol/L NaCl and 30 mmol/L Na-citrate, pH 7.0) at 80°C for 20 minutes followed by thorough washing with 0.15 mol/L PBS. The sections were next treated with pronase (1.0 mg/mL in 0.15 mol/L PBS, Calbiochem, LaJolla, CA) at room temperature for 30 minutes. After this they were rinsed first in 0.15 mol/L PBS and then in buffer A, pH 7.5 (50 mmol/L TRIS HCl, 5 mmol/L MgCl_2 , 10 mmol/L β -mercaptoethanol and 0.005% bovine serum albumin [BSA], fraction V; Sigma Chemical Co, St Louis, MO). Sections were then incubated with ISEL solution prepared in buffer A (0.01 mmol/L deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate) (Promega, Madison, WI), 0.001 mmol/L biotin-11 deoxyuridine triphosphate (Sigma), and 20 U/mL *Escherichia coli* DNA polymerase I (Promega) at 18°C for 2 hours, followed by washing with buffer A and later with 0.5 mol/L PBS (0.5 mol/L NaCl in 0.1 mol/L phosphate buffer, pH 7.5). The specimens were then incubated with avidin-biotin-horseradish peroxidase conjugate (Vectastain Elite ABC Kit-Vector, Burlingame, CA; diluted 1:25 in 0.5 mol/L PBS containing 1% BSA and 0.5% Tween 20). Finally, samples were stained using 0.04% 3,3'-diaminobenzidine tetrahydrochloride diluted in 0.05 mol/L TRIS buffer, pH 7.5, with 0.015% of 30% H_2O_2 for 10 minutes and rinsed well with distilled water. The specimens were then mounted with Fluoromount G (Biotechnology Associate Inc, Birmingham, AL). Slides were left to dry overnight before estimation of PCD.

Simultaneous Assessment of Cell Birth (Proliferation) and Cell Death (Apoptosis) From the Same Biopsy Sample Section Using Double-Labeling by ISEL and Anti-IUdR/BrdU MoAb

For the double-labeling technique designed to simultaneously detect S-phase cells and PCD, sections were first processed for ISEL as described above, and then rinsed thoroughly in 0.5 mol/L TBS (0.5 mol/L NaCl in 0.05 mol/L TRIS buffer, pH 7.5). Subsequently the sections were treated with 4 N HCl for 15 minutes, and the anti-IUdR/BrdU MoAb 3D9 (20-22) diluted 1:200 in 0.5 mol/L TBS containing 0.25% Tween 20 for 60 minutes at room temperature, rabbit antimouse IgG (Dako, Carpinteria, CA) diluted 1:20 in 0.5 mol/L TBS for 30 minutes and with mouse alkaline phosphatase-antialkaline phosphatase antibody (Dako, diluted 1:40 in 0.5 mol/L TBS) for 30 minutes. The sections were rinsed thoroughly with 0.5

mol/L TBS after each of the above-mentioned incubations. Sections were then immersed in the following detection solution for 8 to 10 minutes at 20°C. It contained Naphthol AS-MX phosphate (20 mg) freshly dissolved in 2 mL N,N-dimethylformamide. This was added to 100 mL 0.1 mol/L TRIS buffer, pH 8.2 followed by 0.1 mL 1 mol/L Levamisole and 100 mg of Fast Blue BB salt (Sigma Chemical Co, St Louis, MO). The solution was stirred for 2 minutes and filtered before use. Sections were washed in distilled water and mounted with Fluoromount.

Control Experiments for ISEL Technique

Control experiments for ISEL technique included two types of controls: negative and positive.

Negative Controls

ISEL solution devoid of DNA polymerase was used on control samples as negative controls. These slides were universally negative.

Positive Controls

Positive controls included DNA laddering versus ISEL of fragmented DNA, detection of apoptosis in BM biopsy samples obtained from newly diagnosed high-risk patients with AML, and detection of apoptosis in normal BM biopsy samples obtained from patients with non-Hodgkin's lymphoma (NHL).

DNA laddering versus ISEL of fragmented DNA. HL-60 cells were treated with a topoisomerase inhibitor etoposide (VP 16) at a concentration of 35 $\mu\text{mol/L}$ in RPMI 1640 medium containing 20% fetal bovine serum (FBS). The control cells were incubated in the same medium without etoposide. The incubation was continued for 4 hours at 37°C in 5% CO_2 . At the end of the incubation, each sample was divided into two aliquots. Cells from one aliquot were embedded in plastic, sectioned, and processed for ISEL to determine the percentage of apoptotic cells, whereas the other aliquot was used for DNA extraction. The DNA so obtained was run on an agarose gel containing ethidium bromide.

Detection of apoptosis in BM biopsy samples obtained from newly diagnosed high-risk patients with acute myeloid leukemia (AML). Twenty AML biopsy samples were processed for ISEL before starting remission induction chemotherapy.

Detection of apoptosis in normal BM biopsy samples obtained from patients with NHL. Two NHL patients who had no BM involvement and whose BM biopsy samples were judged by hematopathologists to be indistinguishable from normal BM biopsy samples were also processed for ISEL technique to determine the incidence of apoptotic cell death.

Interpretation of BM Biopsy Specimen Slide

All slides were examined under a light microscope attached to a television screen. At least three different investigators were involved in the interpretation of each slide (A.R. being one of them every time).

Proliferation Studies

Immunohistochemical detection of IUdR- and/or BrdU-labeled S-phase cells was accomplished using either the anti-IUdR/BrdU MoAb 3D9 or the anti-BrdU MoAb Br-3. The nuclei of cells engaged in DNA synthesis were stained dark-brown in single-label slides. The labeling index (LI) was obtained by counting only the labeled myeloid cells as described before.^{23,24} Frequently erythroid islands as well as occasional megakaryocytes were also in S-phase and these were easily recognizable because of their unique morphologic appearance. These labeled erythroid and megakaryocytic cells and their unlabeled counterparts were excluded from the LI calculations. At least 2,000 cells from five different fields were counted.

PCD Studies by ISEL

Coverslips processed for ISEL were mounted onto glass slides and examined on the television screen by a group of observers (AR was always present). The slides were first scanned under low power. ISEL⁺ cells showed clear, punctate brown staining over their nuclei, often being most marked in the perinuclear area. Areas of apoptosis and PCD were identified if these were present in compartmentalized pockets. The following scoring system was used to approximately quantitate the incidence of PCD: absent, only an occasional ISEL⁺ cell, definitely constituting $\leq 15\%$ of all cells; low, up to one third of the biopsy sample containing ISEL⁺ cells; intermediate, between one third and two thirds of the biopsy sample containing ISEL⁺ cells; high, $\geq 75\%$ ISEL⁺ cells.

Simultaneous Proliferation and PCD Studies

In these double-labeled slides, the nuclei of S-phase cells were stained blue, the nuclei of ISEL⁺ cells were stained brown and those S-phase cells that were also ISEL⁺ showed the presence of both brown and blue staining.

Detection of DNA Laddering

Detection of DNA laddering was performed on BM asp cells of six MDS and three AML patients as well as two BM asp samples that were completely normal (obtained from BM transplant donors). After density separation on Ficoll-Hypaque, 1×10^6 cells/mL were incubated for 4 hours at 37°C in 5% CO₂ atmosphere and used for DNA extraction. Cells without the 4-hour incubation were also similarly used for DNA extraction. The reason for incubating cells over various time points before extracting DNA was as follows: Our hypothesis based on the ISEL data in BM biopsy samples was that most of the cells appeared to be in earlier stages of PCD with only low amounts of DNA damage that may not be sufficient to produce laddering in gels. However, leaving the cells in complete medium over specified time points may be enough time for the cells to complete the process of apoptosis with generation of low molecular-weight DNA that would produce the typical DNA laddering. Thus, although initial experiments used a variety of time points (0, 1, 2, 3, and 4 hours) for in vitro incubation of cells in complete medium before DNA extraction, later experiments have used only the 0- and 4-hour time points because they appear to be the two most informative intervals.

Briefly, the cells were washed in 0.1 mol/L PBS, pH 7.5 (0.1 mol/L NaCl in 0.1 mol/L PBS) to remove traces of medium, lysed with guanidine isothiocyanate (GITC) at room temperature and layered on 5.7 mol/L cesium chloride (CsCl) gradient. After overnight centrifugation at 32,000 rpm, the DNA fraction at the interface of CsCl and GITC was aspirated and pooled together with the RNA fraction from the bottom of CsCl gradient tube. Subsequently, this preparation was treated with chilled 100% and 80% ethanol, incubated with Proteinase K (Boehringer Mannheim, Mannheim, Germany) and repeatedly treated with phenol:chloroform:isoamyl alcohol mixture (25:24:1, Sigma) to remove proteins. DNA was then precipitated by adding Na-acetate (3 mol/L, pH 6.0) and double volume of chilled 100% ethanol for 1 hour at -20°C. This DNA fraction was then treated with RNase concentration 1 μ g/mL (final concentration 20 μ g/mL) and subjected to reextraction using phenol:chloroform:isoamyl alcohol mixture followed by treatment with Na-acetate and chilled 100% ethanol as described earlier. Finally, the total DNA content in each sample was estimated by optical density at 260 nm and 5 μ g DNA was loaded in 1.5% agarose gel containing ethidium bromide. After electrophoresis, the gel was photographed under ultraviolet (UV) light.

Statistical Analysis

Mann Whitney tests were used for two sample comparisons of continuous variables. Contingency tables, with chi square statistics or Fisher's exact test, were used for analyzing.

RESULTS

Fifty patients with a confirmed diagnosis of MDS are the subject of this report. The French-American-British (FAB) classification was used to identify the various subtypes of MDS patients.²⁵ There were 19 cases of refractory anemia (RA), 6 cases of refractory anemia with ring sideroblasts (RARS), 17 cases of refractory anemia with excess blasts (RAEB), 7 cases of RAEB in transformation (RAEB-t) and 1 case of chronic myelomonocytic leukemia (CMMOL). BM cellularity was also available in all 50 cases. Table 1 describes the details of FAB type, apoptosis, cell cycle kinetics, biopsy sample cellularity and complete blood counts in all 50 patients.

Results of Control Experiments

A comparison of ISEL technique with DNA laddering detected by gel electrophoresis was performed on HL-60 cells treated with etoposide. Whereas untreated HL-60 cells did not show any low molecular-weight DNA, the cells treated with etoposide showed a characteristic DNA ladder pattern (Fig 1A). Results obtained by ISEL correlated very well with these data. The percentage of apoptotic (ISEL⁺) cells in the control, untreated HL-60 cells was 1.59%, whereas this percentage in etoposide-treated HL-60 cells increased to 13.9% (Fig 1, B and C). The DNA laddering and ISEL methods were similarly compared in at least three more sets of experiments and results obtained repeatedly showed that an accurate detection of apoptotic cells can be accomplished using either. Once satisfied that ISEL is a highly accurate method of detecting PCD, we proceeded to use plastic embedded BM biopsy samples obtained from MDS patients for precise quantitation of apoptotic cell death.

Incidence of spontaneous apoptosis was quantitatively scored in 20 BM biopsy samples obtained from AML patients studied at the time of diagnosis and before receiving any remission induction chemotherapy. None of these biopsy samples showed greater than 10% to 15% of the total cells positive for ISEL staining. The leukemic blasts were universally negative. The few ISEL⁺ cells were usually present in small clusters and appeared morphologically to represent areas of residual normal hematopoiesis. Furthermore, double-labeling showed that none of the S-phase cells were ISEL⁺.

PCD was also studied in two normal BM biopsy samples obtained from NHL patients. Both the patients showed geographically well-defined areas of apoptotic cell death scattered throughout the samples. Within these "geographically restricted islands of death," all three lineages of cells were ISEL⁺, as were occasional stromal cells. On the whole, such areas accounted for approximately one third of the BM biopsy sample area, but it was never as extensive as in MDS patients. Finally, S-phase cells were never double-labeled for ISEL in these samples.

Proliferation Studies

Sequential labeling of S-phase cells was performed in vivo using infusions of IUdR and/or BrdU. Labeling indices (LI) from BM biopsy samples were available in 46 patients. The median LI was 28.6% with a range of 13% to 49.1%. Dura-

Table 1. Cell Cycle Kinetics, PCD, and Clinical Characteristics of Patients With MDS

S No.	Patient GMA No.	FAB	ISEL	LI %	Ts (hs)	TC (hs)	Cellularity (%)	WBC Count (x10 ⁹ /L)	Hb Count (g/dL)	Platelets Count (x10 ⁹ /L)
1	207/88	RA	High	39.6	15.6	39	30	4.5	11.6	92
2	131/90	RAEB-t	High	47	19	40	15	1.7	12.3	182
3	130/89	RA	High	26	NA	NA	90	8.4	10.6	433
4	250/87	RA	High	25.1	9.7	38.6	50	2.7	10.1	68
5	14/87	RAEB	High	21.3	15.2	17.3	95	12.8	7.4	44
6	3/87	RA	High	15	7.4	47	10	3.9	10.1	29
7	268/90	RARS	High	29.3	65.8	224.6	90	38.1	8.2	360
8	215/90	RARS	High	13	8.06	62	90	13	9.6	409
9	21/91	RARS	High	37	6.72	18.16	40	4.5	11.8	71
10	43/93	RAEB	High	24	NA	NA	20	0.7	6.1	24
11	11/93	RAEB	High	26	NA	NA	60	3.7	9.3	127
12	5/93	RAEB	High	18	NA	NA	20	1.6	9.2	225
13	63/93	RA	High	25	NA	NA	70	2.9	10.1	256
14	169/90	RAEB-t	High	32	NA	NA	70	2.5	9.3	61
15	616/87	RAEB-t	High	31	22	71	60	NA	NA	NA
16	390/87	RAEB	High	33.6	14.7	43.7	80	14.6	12	219
17	19/93	RAEB-t	High	18	NA	NA	70	10.1	8.4	15
18	21/93	RAEB	High	18	NA	NA	40	1.5	9.1	45
19	20/91	RARS	High	30.6	11.82	38	40	7.5	8.4	417
20	86/94	RA	High	24	NA	NA	80	3.1	10.6	63
21	98/94	RAEB	High	28.7	4.6	16.1	70	4.7	7.9	224
22	150/94	RA	High	31.5	27.9	88.6	80	2.5	11.1	162
23	64/94	RAEB-t	High	28.5	5.6	19.8	40	1.3	8	25
24	130/94	RAEB	High	24.5	24.9	101.8	40	25.7	9.3	43
25	281/94	RAEB	High	18.7	5	27	70	9.8	9.4	99
26	88/94	RAEB	High	23.9	3.6	15	90	1.6	8.3	56
27	382/88	RA	Intermediate	46.6	NA	NA	35	NA	NA	NA
28	128/90	RARS	Intermediate	49.1	6.8	13.8	90	1.4	8.3	43
29	34/89	RA	Intermediate	39	24	61.5	65	15.2	9.2	729
30	100/89	RAEB-t	Intermediate	39.3	17.5	44.5	50	4.9	9.2	32
31	182/90	RARS	Intermediate	32.3	16.2	50.15	70	3.1	8	11
32	609/87	RAEB-t	Intermediate	31	22	71	50	4	8.8	53
33	136/90	RAEB	Intermediate	29	32.3	111.3	60	6.7	8.2	196
34	292/94	RAEB	Intermediate	33.8	6.9	20.4	80	3.2	9.1	93
35	390/87	RA	Low	33.1	13.8	41.7	55	1	8.7	212
36	74/87	RA	Low	25.3	14.6	57.5	50	3.1	10.5	70
37	25/93	RA	Low	41	NA	NA	70	8.7	9.3	52
38	46/93	RA	Low	16	NA	NA	30	1.7	7.4	79
39	103/93	RAEB	Low	38	NA	NA	70	0.8	8.8	28
40	26/91	RA	Low	35	5.8	16.5	80	3.4	7.8	104
41	112/94	RA	Low	NA	5.3	NA	60	3.7	13.2	126
42	23/94	CMMOL	Low	19.1	NA	NA	60	11.8	6.5	173
43	104/94	RA	Low	NA	NA	NA	30	6.3	9.9	30
44	48/94	RAEB	Low	NA	NA	NA	60	6.3	12.8	915
45	245/94	RA	Low	23.9	3	12.6	30	2.5	6.7	52
46	51/93	RAEB	Negative	14	NA	NA	50	3.1	12.9	250
47	68/87	RAEB	Negative	24	NA	NA	70	2.5	9.2	1,551
48	282/94	RAEB	Negative	24.9	5.8	23.1	80	7.4	10	90
49	275/94	RA	Negative	44.5	4.1	9.1	10	1.1	8.1	29
50	88/93	RA	NA	NA	NA	NA	1	4	10.9	35

Abbreviations: GMA, glycol methacrylate biopsy; WBC, white blood cell; Hb, hemoglobin; NA, not available.

tion of S-phase (Ts) was available in 30 patients with a median of 12.8 hours and a range of 3.0 to 65.8 hours. Total cell cycle time (Tc) was available in 29 with a median of 40.0 hours (range, 9.1 to 224.6 hours). These data are also provided in Table 1 for individual patients.

Apoptosis Studies

In situ end labeling of fragmented DNA was performed on the plastic embedded biopsy samples of all 50 patients.

A distinct brown staining in a variety of patterns over the nucleus identifies a cell as being engaged in DNA cleavage. Among the 50 cases being reported, one biopsy sample was excluded because of poor quality of the sample whereas four cases showed "absent" apoptosis or only occasional ISEL⁺ cells and were considered as ISEL⁻ (Table 1). Of the remaining 45 cases, 26 had high, 8 had intermediate and 10 had low ISEL-positivity.

Interestingly, all three lineages of hematopoietic cells in-

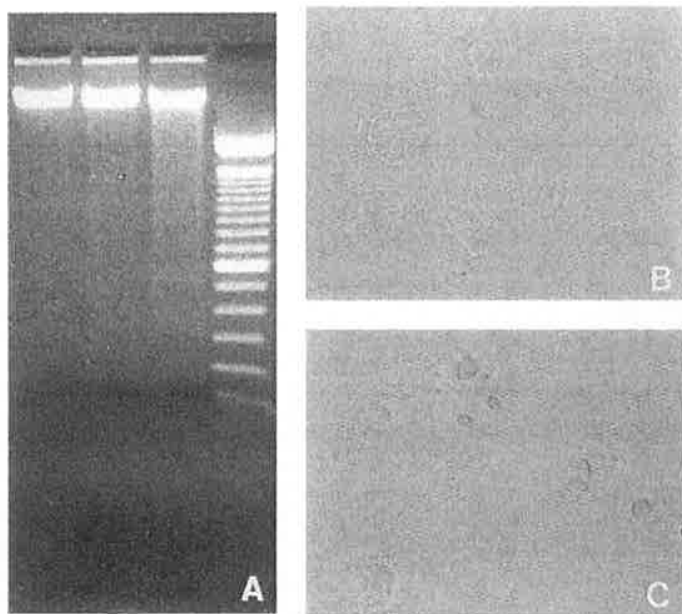


Fig 1. (A) Agarose gel electrophoresis of DNA extracted from HL-60 cells incubated with etoposide at varying concentrations. Lane 1, no etoposide; lane 2, treated with etoposide at 17.5 $\mu\text{mol/L}$ for 4 hours; lane 3, treated with etoposide at 35 $\mu\text{mol/L}$ for 4 hours; lane 4, DNA molecular-weight marker, 100-bp DNA ladder (GIBCO Life Technologies, Grand Island, NY). Note the appearance of laddering in lane 3. (B) In situ end labeling on control HL-60 cells without etoposide treatment show no positive staining. (C) ISEL of etoposide-treated HL-60 cells show distinct ISEL staining over clearly karyorrhectic cells.

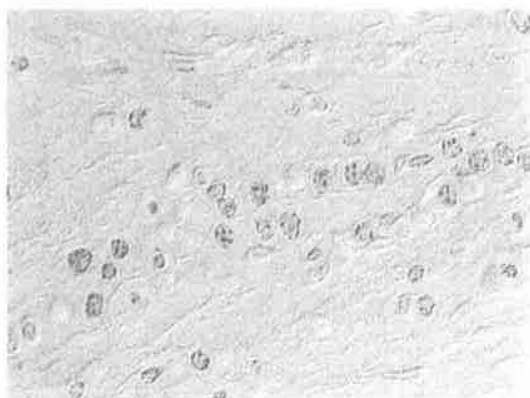


Fig 2.

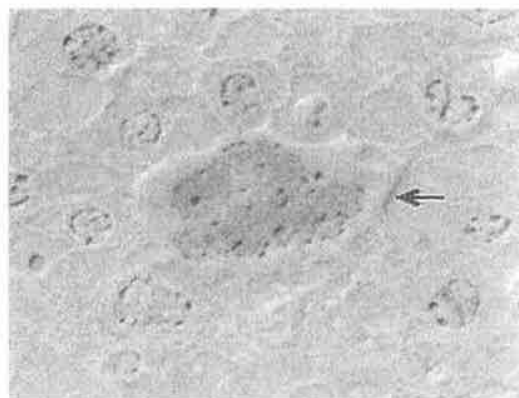


Fig 4.

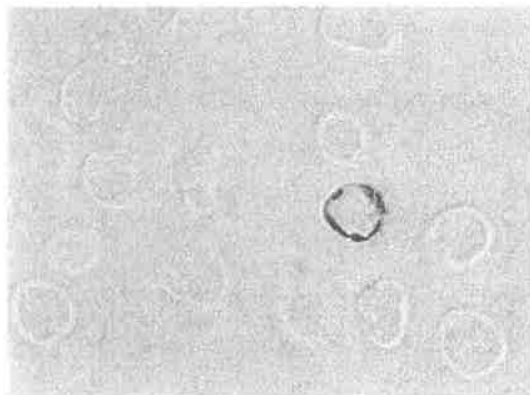


Fig 3.

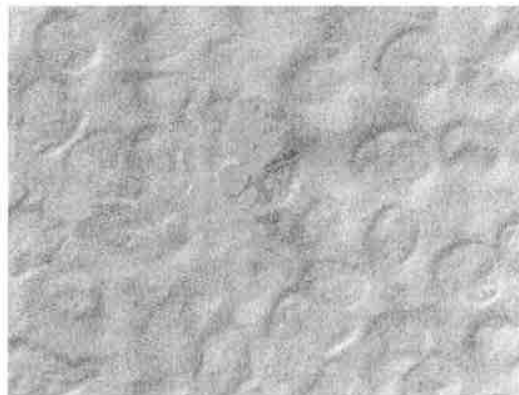


Fig 5.

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