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1-β-D-Arabinofuranosylcytosine-, mitoxantrone-, and paclitaxel-induced apoptosis in HL-60 cells: improved method for detection of internucleosomal DNA fragmentation

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Abstract. We investigated the ability of different doses and durations of exposure to the chemotherapeutic drugs 1-β-D-arabinofuranosylcytosine (Ara-C), mitoxantrone (MTN), and paclitaxel (taxol, TXL) to induce internucleosomal DNA fragmentation and apoptosis in human acute myeloid leukemia (AML) HL-60 cells in suspension culture. At clinically achievable concentrations, all three drugs have been shown to induce apoptosis in HL-60 cells. An improved method was developed for the isolation of pure genomic DNA and the detection of drug-induced internucleosomal DNA fragmentation in <1.0 μg of DNA sample by agarose gel electrophoresis. Morphologic evidence for apoptosis was determined by light microscopy following Wright staining, and cell viability was assessed by trypan blue dye exclusion. Internucleosomal DNA fragmentation was observed following exposure to 1.0 μ*M* Ara-C for 4 h, which increased with 10 and 50 μ*M* Ara-C. Incubation with 100 μ*M* Ara-C produced internucleosomal DNA fragmentation starting at 3 h, which increased with longer periods of exposure to Ara-C. Utilizing a schedule of 1-h exposure followed by 3-h suspension in drug-free medium, 0.25 μ*M* MTN was found to initiate DNA fragmentation, which increased with exposure to 1.0 and 5.0 μ*M* MTN. However, identical treatment with higher concentrations of MTN resulted in random DNA degradation. Alternatively, continuous exposure to 1.0 μ*M* MTN for 3 h was necessary to initiate internucleosomal DNA fragmentation. This increased with exposure intervals of up to 6 h. Exposure to TXL concentrations as low as 0.01 μ*M* for 24 h caused internucleosomal DNA fragmentation, which increased with dose escalation (0.05, 0.1, 0.5, and 1.0 μ*M*) of TXL. Although continuous exposure to 1.0 μ*M* TXL for a period as short as 8 h produced internucleosomal DNA fragmentation, this increased significantly with longer exposure intervals. In general there appears to be a threshold concentration and duration of exposure below which none of these three drugs activates endonucleolytic internucleosomal DNA fragmentation and apoptosis. This threshold is lower for the DNA-interactive drugs MTN and Ara-C but higher for the non-DNA-interactive drug TXL. Higher doses or prolonged treatments with the drugs produce random DNA fragmentation associated with necrotic cell death. These in vitro results may further improve our understanding of the antileukemic cytotoxic effects of these drugs, which may enable a more rational design of drug regimens for optimal treatment of AML.

Key words: Apoptosis – DNA fragmentation – HL-60 cells – Ara-C – Mitoxantrone – Taxol

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Introduction

Programmed cell death (PCD), or apoptosis, is an active and gene-directed form of cell death, which has recently been shown to be induced in human myeloid leukemia cells by a variety of anticancer drugs [4, 20, 25]. The morphologic features of apoptosis are cell shrinkage, cell-surface blebbing, cytoplasmic condensation, and nuclear compaction followed by breakdown of the nucleus into small fragments [42]. The best-identified biochemical characteristic of apoptosis is the induction of double-strand DNA fragmentation at linker regions between nucleosomes, resulting in internucleosomal DNA fragments of multiples of 180–200 bp in size [17]. This internucleosomal cleavage is caused by either the activation or the de novo synthesis of an endonuclease [2]. Agarose gel electrophoresis displays the internucleosomal DNA fragments from apoptotic cells in a typical ladder pattern, whereas the DNA cleavage in necrotic cells is random and is seen as a smear [2, 17]. Cells undergoing apoptosis are unlikely to repair their DNA by religation of the two correct ends of all the double-strand breaks of the DNA fragments, and the cells ultimately die [21]. Although DNA fragmentation may not always be

evident in all instances of apoptosis, in most cases it is a biochemical hallmark of apoptotic cell death [28].

Ara-C is an effective chemotherapeutic agent that is widely used for the treatment of acute myelogenous leukemia (AML) [14]. Intracellularly, Ara-C is sequentially phosphorylated to cytosine arabinoside triphosphate (Ara-CTP), which is a potent inhibitor of DNA synthesis by its direct incorporation into DNA [13, 29]. Exposure to high concentrations of Ara-C results in the saturation of intracellular Ara-CTP accumulation, higher Ara-C DNA incorporation, and DNA strand breaks [10]. In addition, high doses of Ara-C have been shown to induce internucleosomal DNA fragmentation and apoptosis in AML cells [24]. Mitoxantrone (MTN) is an anthracenedione derivative with promising antileukemic activity [1, 3]. Intracellularly, it is known to intercalate between base pairs of the DNA helix as well as to bind to DNA by nonintercalative electrostatic interaction [11]. MTN treatment produces single- and double-strand breaks in DNA [26]. Some of the protein-associated DNA strand breaks result from a cleavable complex with topoisomerase II, an enzyme whose activity is markedly inhibited by MTN [18]. High concentrations of MTN ($\geq$1.0 μmol/l) exhibit a steep dose-to-inhibition of clonogenic survival relationship toward leukemic blasts [23]. Recently, high-dose MTN has been shown to induce apoptosis in human myeloid leukemia cells [7]. Taxol (TXL) is a diterpenoid plant product that has been shown to possess antileukemic activity [35, 39]. Unlike other antimicrotubule agents (colchicine, vincristine, and vinblastine) that cause microtubule disassembly, TXL promotes all aspects of microtubule assembly from tubulin dimers, resulting in stable, nonfunctional microtubule bundles and cell-cycle growth arrest in the G_2/M phase [31, 34]. Although it is not a DNA-interactive agent, TXL has also recently been demonstrated to induce endonucleolytic DNA fragmentation and apoptosis in human leukemia cells [8].

As noted above, since the antileukemic drugs Ara-C, MTN, and TXL all induce the gene-directed and active form of apoptotic cell death in human myeloid leukemia cells, the recent focus in cancer chemotherapy has been to examine the optimal dose and schedule of the drugs that would produce internucleosomal DNA fragmentation and apoptotic cell death of leukemic cells. In the present in vitro studies, we developed an improved method for the detection of internucleosomal DNA fragmentation by agarose gel electrophoresis and used this method to determine the optimal dose and schedule of Ara-C, MTN, and TXL that would induce apoptosis in HL-60 cells.

Materials and methods

Drugs and other chemicals

Ara-C was purchased from Sigma Chemical Co. (St. Louis, Mo.). It was stored as a powder at 4° C and was freshly prepared by dissolving it in medium and sterilizing it through a 0.22-μm filter (Millipore, Cambridge, Mass.). MTN was obtained from Lederle, Inc. (Carolina, Puerto Rico). It was stored at room temperature and was appropriately diluted in medium immediately before the use. TXL was a kind gift from Dr. Patricia A. Pilia of NaPro Biotherapeutics Inc. (Boulder, Col.). A stock solution (10 m*M*) of TXL was prepared in 100% dimethylsulfoxide (DMSO), and small aliquots of this stock were stored at 4° C. It was diluted in medium to achieve the desired concentrations. In no experiment did the DMSO concentration exceed 0.01%. By itself, this concentration of DMSO does not produce internucleosomal DNA fragmentation in HL-60 cells. RNases A and T1 were purchased from Sigma Chemical Co. (St. Louis, Mo.). Proteinase K and ultrapure DNA-grade agarose were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and Bio-Rad Laboratories (Hercules, Calif.), respectively. All other chemicals were of analytical grade.

Cells and culture

HL-60 cells were freshly grown as a suspension culture in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) and antibiotics at 37° C in a humidified atmosphere containing 5% CO_2 [16]. The viability of the cells was assessed by their ability to exclude 0.5% trypan blue dye. Cell density in the culture was determined by a Coulter counter (Model ZM; Hialeah, Fla.). Only exponentially growing, viable cells were used for the studies. Cells were seeded at a final density of 0.5×10^6 cells/ml in all experiments and were treated with drug at the designated dose and exposure interval. After drug treatment, when necessary, cells were washed twice with phosphate-buffered saline (PBS, pH 7.3) and reincubated in drug-free medium.

Morphology of apoptotic cells

After treatment with or without drug, 50×10^3 cells were washed with PBS (pH 7.3) and resuspended in the same buffer. Cytospin preparations of the cell suspensions were fixed and stained with Wright stain. Cell morphology was determined by light microscopy. In all, 5 different fields were randomly selected for counting of at least 500 cells. The percentage of apoptotic cells was calculated for each experiment. Cells designated as apoptotic were that which displayed the characteristic morphologic features of apoptosis, including cell-volume shrinkage, chromatin condensation, and the presence of membrane-bound apoptotic bodies. The assessment of the percentage of apoptotic cells was confirmed by an additional independent observer who was blinded to the results obtained by the first observer.

Genomic DNA extraction

Following incubations with each drug at the designated concentrations and schedules, 1×10^6 cells were pelleted in a 1.5-ml Eppendorf tube by centrifugation at 1,000 rpm for 5 min and then washed twice with PBS (pH 7.3). Cells were gently resuspended in 50 μl of the lysis buffer [200 m*M* NaCl, 10 m*M* TRIS-HCl (pH 8.0), 40 m*M* ethylenediaminetetraacetic acid (EDTA, pH 8.0), 0.5% sodium dodecyl sulfate (SDS), 200 ng RNase A/μl, 10 U RNase T1/μl] and incubated at 37° C for 1 h. To the cell lysate was added 200 μl of the digestion buffer [200 m*M* NaCl, 10 m*M* TRIS-HCl (pH 8.0), 0.5% SDS, 125 ng proteinase K/μl]. The contents were mixed by inversion several times and then incubated at 50° C for 3 h. An equal volume of a mixture of phenol (pH 8.0) and chloroform (1:1, v/v) was added, gently mixed for 10 min, and stored at room temperature for 2 min. The two phases were separated by centrifugation at 3,000 rpm for 10 min. The viscous aqueous phase (not the interphase) was transferred to a fresh tube with a wide-bore pipette tip. The phenol/chloroform extraction was repeated. The aqueous phase was extracted with an equal volume of chloroform. To enhance the precipitation of fragmented DNA (if any), 1.0 *M* $MgCl_2$ was added to the aqueous phase to a final concentration of 10 m*M*. The total DNA was precipitated by the addition of 2 vols. of absolute ethanol with several inversions. DNA was pelleted by centrifugation at 3,000 rpm for 15 min, washed twice with 70% ethanol, and air-dried. The pellet was dissolved in 25 μl of 10 m*M* TRIS-HCl

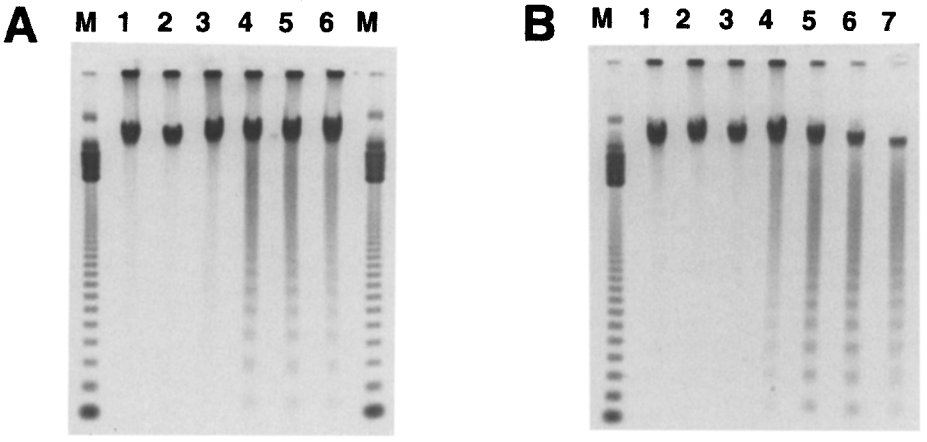

Fig. 1 A, B. Internucleosomal DNA fragmentation in HL-60 cells by Ara-C. An equal amount (600 ng) of genomic DNA/lane was loaded in the gel. **A** Dose response to Ara-C treatment for 4 h (*M* 123-bp ladder, *lanes 1–6* DNA from cells treated with different doses of Ara-C: 0, 0.1, 1.0, 10, 50, and 100 μ*M*, respectively). **B** Response to different intervals of exposure to 100 μ*M* Ara-C (*M* 123 bp ladder, *lanes 1–7* DNA from cells treated with Ara-C for 0, 1, 2, 3, 4, 8, and 16 h, respectively)

(pH 8.0) and 1.0 m*M* EDTA (pH 8.0). The concentration and purity of the DNA was determined spectrophotometrically.

Agarose gel electrophoresis

Completely molten agarose (1.8%, w/v) in TAE (40 m*M* TRIS, 40 m*M* sodium acetate, 1.0 m*M* EDTA; pH 8.3) was poured on the tray to make a 1.5-mm thick gel. The agarose gel was solidified and dried for 1 h at room temperature and the gel was transferred to a Mini-Sub cell (Bio-Rad) containing 1xTAE (pH 8.3). The volume of the running buffer was adjusted to a 1.0-mm depth from the surface to the submerged gel, and electrophoresis was prerun at 2 V/cm for 1 h. Wells were cleaned by flushing the running buffer in and out with a pipette tip. Samples were prepared by mixing the DNA (<1.0 μg) with a 0.2 vol. of loading dye mixture (0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose), after which they were heated at 60° C for 3 min, rapidly chilled in ice water, and loaded onto the gel. The samples were subjected to electrophoresis at 2 V/cm for 3.5 h in ice-cold conditions. The gel was stained with ethidium bromide (0.5 μg/ml) for 15 min with slow rocking and destained in water until the background fluorescence disappeared. DNA-intercalated ethidium fluorescence was observed on a UV (302 nm) transilluminator and photographed on Polaroid film 665 (P/N) using an orange filter. Subsequently, the negative was developed for the DNA profile.

Statistical analysis

Significant differences between values obtained in a population of HL-60 cells treated under different experimental conditions were determined by paired *t*-test analyses.

Results

Internucleosomal DNA fragmentation, the biochemical hallmark of apoptosis, was verified by an improved method of agarose gel electrophoresis of genomic DNA from cells treated at different doses and schedules of Ara-C (Fig. 1), MTN (Fig. 2) and TXL (Fig. 3). The method was sensitive enough to detect the "ladder" pattern of internucleosomal DNA fragmentation in 400–800 ng of the electrophoresed genomic DNA sample. The method also allowed for the accurate comparison of the amount of fragmented DNA in each lane of the same gel, since the identical amount of genomic DNA purified from cells treated under different conditions was loaded into the well of each lane.

Figure 1A demonstrates that maximal DNA fragmentation occurred following a 4-h treatment with 10 μ*M* Ara-C. No further increase in DNA fragmentation was noted despite an increase in the dose of Ara-C from 10 to 100 μ*M* (Fig. 1A, lanes 4–6). Although even a 3-h exposure to 100 μ*M* Ara-C resulted in internucleosomal DNA fragmentation (Fig. 1B, lane 4), this increased when the exposure interval was increased to 4 and 8 h (Fig. 1B, lanes 5 and 6) but slightly decreased following exposure to 100 μ*M* Ara-C for 16 h (Fig. 1B, lane 7). The different amounts of fragmented DNA noted following treatment at an identical dose of and interval of exposure to Ara-C (100 μ*M* for 4 h), as shown in Fig. 1A, lane 6, versus Fig. 1B, lane 5, were due to the variability of ethidium bromide staining and destaining as well as due to the slight

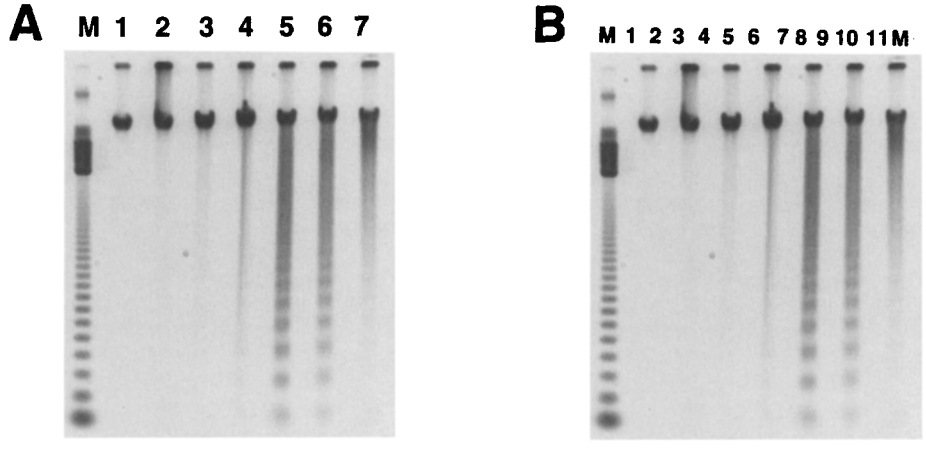

Fig. 2 A, B. MTN-mediated internucleosomal DNA fragmentation in HL-60 cells. Each lane contains 400 ng of genomic DNA. **A** Dose response to MTN treatment for 1 h followed by incubation of the cells for 3 h in drug-free medium (*M* 123-bp ladder, *lanes 1–7* DNA from cells treated with different doses of MTN: 0, 0.01, 0.1, 0.25, 1.0, 5, and 20 μ*M*, respectively). **B** Response to different intervals of exposure to 1.0 μ*M* MTN (*M* 123-bp ladder, *lanes 1–11* DNA from cells treated with MTN for 0, 0.25, 0.5, 1.0, 2, 3, 4, 5, 6, 7, and 8 h, respectively)

Table 1. Apoptosis following treatment with different doses of and schedules of exposure to Ara-C, MTN, and TXL[a]

Drug	Dose (μ*M*)	Mean % apoptotic cells[b]	Drug	Time (h)	Mean % apoptotic cells[b]
Ara-C	0	2.0	100 μ*M* Ara-C	0	2.0
	0.1	2.5		1	6.6
	1.0	8.7*		2	10.0
	10.0	21.3*		3	13.8
	50.0	21.8		4	21.6*
	100.0	18.7		8	19.7
				16	15.1
MTN	0.01	3.5	1 μ*M* MTN	1	7.0
	0.1	5.5		2	7.4
	0.25	10.5*		3	8.5
	1.0	18.1*		4	16.0
	5.0	16.7		5	20.0
	20.0	8.8*		6	35.8*
				7	29.0
				8	28.0
TXL	0.01	10.8	1 μ*M* TXL	1	4.2
	0.05	26.4*		4	13.2
	0.1	27.4		8	13.3
	0.5	28.2		16	23.8*
	1.0	33.1		24	29.3
				48	32.9

* Values significantly greater than those listed immediately above ($P < 0.05$)

[a] Drug treatment time: Ara-C, 4 h; MTN, 1 h followed by 3 h without drug; TXL, 24 h

[b] Mean of 3 separate experiments

variation in the thickness of the two gels that can occur on 2 separate days of experimentation. Also, the reduced amounts of unfragmented genomic DNA observed in the wells of lanes 5–7 in Fig. 1B may have been due to a more complete endonucleolytic fragmentation of DNA into 200-bp integer multiples secondary to the prolonged treatment with 100 μ*M* Ara-C. The morphologically detectable mean percentages of apoptotic cells attributable to Ara-C treatment are presented in Table 1. The data reveal that a 4-h exposure to Ara-C concentrations above 10 μ*M* did not enhance the percentage of cells displaying morphologic features of apoptosis ($P > 0.05$). Also, increasing the duration of exposure to 100 μ*M* Ara-C from 4 to 16 h led to a decline in the morphologic evidence of apoptosis in HL-60 cells (Table 1).

Following treatment with different doses of MTN for 1 h, HL-60 cells were washed free of the drug and kept in drug free medium for 3 h, after which DNA fragmentation was determined (Fig. 2A). Exposure to 1.0 and 5.0 μ*M* MTN produced internucleosomal DNA fragmentation (Fig. 2A, lanes 5 and 6), which declined with higher doses of MTN (Fig. 2A, lane 7). DNA extraction and agarose gel electrophoresis immediately after treatment with 1.0 μ*M* MTN for increasing intervals, without the subsequent 3-h suspension in drug-free medium, demonstrated that internucleosomal DNA fragmentation are first discernable after 3 h (Fig. 2B, lane 7) and was absent when evaluated immediately after 1 h of MTN treatment (Fig. 2B, lane 4). Taken together, these findings indicate that after exposure to MTN, a finite period (2–3 h) is necessary for the optimal endonuclease activation and induction of internucleosomal

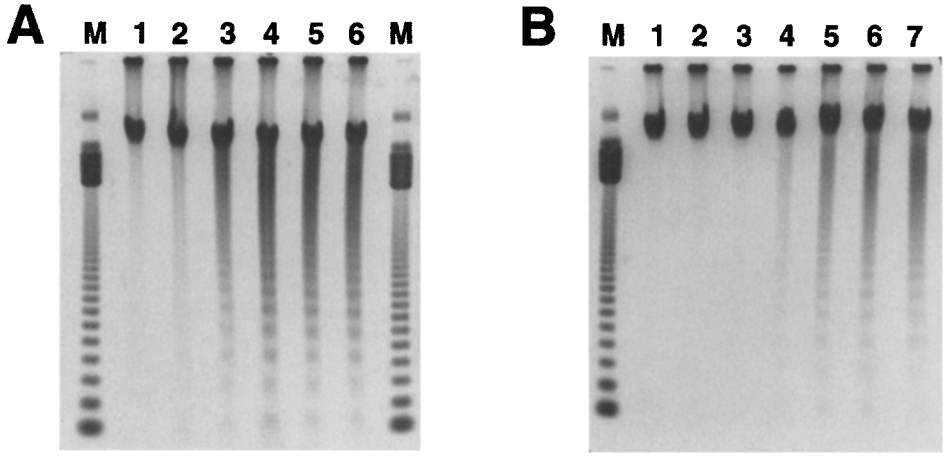

Fig. 3 A, B. TXL-induced internucleosomal DNA fragmentation in HL-60 cells. Each lane was loaded with 800 ng of genomic DNA. **A** Dose response to TXL treatment for 24 h (*M* 123-bp ladder, *lanes 1–6* DNA from cells treated with different doses of TXL: 0, 0.01, 0.05, 0.1, 0.5, and 1.0 μ*M*, respectively). **B** Response to different intervals of exposure to 1.0 μ*M* TXL (*M* 123-bp ladder, *lanes 1–7* DNA from cells treated with TXL for 0, 1, 4, 8, 16, 24, and 48 h, respectively)

DNA fragmentation. Morphologic evaluation of apoptosis also revealed that the percentage of apoptotic cells did not increase markedly following treatment with MTN concentrations above 5.0 μ*M*. In contrast, increasing the duration of continuous exposure to 1.0 μ*M* MTN to up to 6 h produced an increase in the percentage of apoptotic cells (Table 1).

Although TXL is a non-DNA-interactive agent, exposure to ≥0.05 μ*M* TXL for 24 h, as shown in Fig. 3A, lanes 3–6, induced significant endonucleolytic DNA fragmentation in HL-60 cells. However, the amount of DNA fragmentation did not significantly increase with higher concentrations of TXL (0.5 and 1.0 μ*M*). This finding was also supported by the presence of only a modest increase in the percentage of apoptotic cells following exposure to >0.05 μ*M* TXL as detected by the morphologic evaluation of Wright-stained, cytospun preparations of drug-treated cells (Table 1). Continuous exposure to 1.0 μ*M* TXL for increasing intervals demonstrated that internucleosomal DNA fragmentation was discernable only after 16 h of treatment, with a further modest increase occurring after exposure intervals of up to 48 h (Fig. 3B, lanes 5–7). Once again, these results were corroborated by the finding of a modest increase in the percentage of morphologically detectable apoptotic cells when the exposure interval was increased from 24 to 48 h (Table 1).

Discussion

In the present report, we describe an improved method for the isolation of genomic DNA from drug-treated cells and for the detection of DNA fragmentation by agarose gel electrophoresis. Isolation of DNA was based on a simple protocol involving few steps. The DNA yield was about 20 μg/10^6 HL-60 cells, and the preparation was free of protein (A_{260}/A_{280}, ≥1.9) and RNA (Figs. 1–3). On a typical gel (1.5 mm thick) stained with ethidium bromide, the internucleosomal DNA-fragmentation pattern was detectable when 400 ng DNA was loaded in the well. At this amount of DNA, about 1.6–2.0 × 10^3 apoptotic cells should have their DNA to detect internucleosomal DNA fragmentation. We also found that a gel thickness of >2.0 mm abolished this high sensitivity of detection of internucleosomal DNA fragmentation. The resolution of high-molecular-weight DNA fragments was better on a 1.8% agarose gel than in gels with a lower concentration of agarose. A higher agarose concentration (>1.8%) improved this resolution but impaired the ability to make a gel of optimal thickness. The internucleosomal DNA-fragmentation pattern became particularly sharp when electrophoresis was performed at 2 V/cm under ice-cold conditions.

There have been other reports for the detection of drug-induced internucleosomal DNA fragmentation in genomic DNA by agarose gel electrophoresis [4, 30, 37]. However, these methods could detect a fragmentation pattern only when higher amounts (10–20 μg/lane) of DNA were loaded per well. Therefore, our method appears to be at least 20–50 times more sensitive than the other methods reported for nonradioactive detection of internucleosomal DNA fragmentation. Our new method of genomic DNA extraction coupled with the thin-gel electrophoresis technique provided a sensitivity comparable with that described for a recently reported method utilizing ^{32}P labeling of the nucleosomal fragments [27]. However, our method has the advantage of not having to deal with radioactivity. Our improved method is also inexpensive, as it does not require large volumes of cell culture. Moreover, our method can be modified for the quantitation of DNA fragmentation. For this purpose, the DNA-intercalated ethidium fluorescence on the gel can be electronically imaged and analyzed to determine the percentage of DNA fragmentation after drug treatment.

A variety of anticancer drugs with disparate intracellular mechanisms of action have been demonstrated to engage the final common pathway of endonucleolytic DNA fragmentation and apoptotic cell death [19, 20]. This finding has attracted widespread attention because apoptosis is a gene-directed, active form of cell death that is normally operative physiologically in organogenesis during embryonic development and, unlike cell necrosis, is not attended with an untoward inflammatory host response [40]. In addition, the possibility exists that modulation of its mechanism or of signals that control drug-induced apoptosis would improve the antileukemic effects of known chemotherapeutic agents [5, 7, 12]. To this end, as a first step, a careful examination of the ability of clinically relevant doses and schedules of three antileukemic drugs to induce apoptosis was carried out. The present in vitro studies evaluated the various doses and exposure intervals of Ara-C, MTN, and TXL that produce internucleosomal DNA fragmentation as determined by a highly sensitive assay utilizing <1.0 μg of DNA sample. The evidence for apoptotic cell death was further confirmed by morphologic examination of drug-treated cells. Our results highlight that there is a threshold dose and exposure interval for each of the three drugs, above which endonucleolytic DNA fragmentation is activated. These thresholds varied according to the drug tested. In addition, with each drug, increasing the dose or exposure interval had a disparate effect on the amount of internucleosomal DNA fragmentation and the percentage of apoptotic cells.

The intracellular target of MTN and Ara-C is DNA, whereas TXL interacts with tubulin [26, 29, 31]. This difference in intracellular targets may explain why MTN and Ara-C induce internucleosomal DNA fragmentation and apoptosis after short intervals of exposure (≤4 h), whereas 16 h of exposure to TXL is needed for similar effects with respect to apoptosis (Figs. 1–3). This observation is also supported by previous reports about other DNA-damaging drugs such as nitrogen mustard, cisplatin, etoposide, and camptothecin, which produce rapid endonucleolytic internucleosomal DNA fragmentation [4, 32, 36, 38]. Despite the relative delay in the induction of apoptosis, clinically achievable concentrations and schedules of taxol, as also previously reported, were as effective as MTN and Ara-C in mediating apoptosis [8]. Our data also show that in general, shorter intervals of exposure to each of the three drugs were less effective in inducing apoptotic cell death in HL-60 cells. It is also noteworthy that immediately after

exposure to clinically relevant doses and schedules of Ara-C, MTN, and TXL, the biochemical or morphologic features of apoptosis were not detectable in the majority of HL-60 cells. The resistance of these cells to apoptosis may have been due to an impaired interaction between the drug and its intracellular target or to impaired molecular signaling and activation of endonucleolytic DNA fragmentation leading to apoptosis [6, 22, 38]. Although p26BCL-2 is the prototype, differential expression of a variety of other intracellular oncoproteins may retard drug-induced endonucleolytic DNA fragmentation and apoptosis and may be responsible for protecting the majority of HL-60 cells from apoptosis [38, 41]. These include the gene products of *bcl-*$_{XL}$, *bax* and mutant *p53* genes [9, 15, 33]. However, the mechanisms by which these gene products confer resistance to apoptosis have not yet been elucidated.

In conclusion, the present in vitro data may improve our understanding of the relationship between clinically relevant doses and intervals of exposure to Ara-C, TXL, and MTN with regard to their ability to induce internucleosomal DNA fragmentation and apoptosis in leukemic cells. These results may help to improve further the clinical application of these drugs for controlling acute myeloid leukemia by achieving the appropriate concentrations of the drugs for the desired period in plasma to induce apoptotic death of the leukemic cells.

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