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Apoptosis without decrease of cell DNA content

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Abstract Apoptosis of human B cells and murine T and B cells was analyzed by DNA agarose gel electrophoresis, clamped homogeneous electric field, measurement of cell DNA content by flow cytometry, transmission electron microscopy and by UV microscopy. Apoptosis was induced by etoposide (an inhibitor of topoisomerase II), by the calcium ionophore ionomycin or by cross-linking of membrane immunoglobulins (Ig) with anti-Ig-antibodies. Two types of apoptosis could be defined. Apoptosis resulting in small DNA fragments (180–200 base pairs and multiples thereof) was associated with a typical 'ladder' in agarose gel electrophoresis and a decrease in cell DNA content assessed by flow cytometry. Conversely apoptosis with large DNA fragments (100–150 kilobase pairs) was only demonstrated by clamped homogeneous electric field but was not associated with decreased cell DNA content or the observation of DNA ladders. Nuclear condensation without fragmentation was more frequent when apoptosis generated large DNA fragments. The type of apoptosis appears to be an intrinsic property of each cell type.

Key words: Apoptosis; Decrease of DNA content; DNA fragmentation; Etoposide

1. Introduction

Programmed cell death or apoptosis is characterized by typical morphological alterations demonstrated by transmission electron microscopy, including condensation of the chromatin in tight apposition to the nuclear envelope, alteration of the nuclear envelope and fragmentation of the nucleus, in contrast with conserved integrity of the plasma membrane and organelles [1,2]. A typical feature of cells undergoing apoptosis is the diminution of cell DNA content which can be demonstrated as the occurrence of a 'Sub-G1' cell population by flow cytometric analysis of DNA content [3]. Cell death by apoptosis has been described as the result of the activation of Ca^{2+}-dependent endonucleases which split double stranded DNA at exposed sites between nucleosomes, generating fragments of 180–200 base pairs and multiples thereof [4]. Agarose electrophoresis of cellular DNA showing a typical aspect of 'ladder' was proposed as the reference method for demonstrating apoptosis [5–8]. Recently, Oberhammer et al. [9] described apoptotic death in epithelial cells with cleavage of DNA to 300 and/or 50 kb fragments prior to, or in absence of, internucleosomal fragmentation.

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In this study we investigated the occurrence of small or large DNA fragments in several cell lines undergoing apoptosis. We studied whether the decrease of cell DNA content was associated with the type of DNA fragmentation or with any of the morphological alterations. We report that only cells generating small DNA fragments during apoptosis manifest a decrease in cell DNA content.

2. Materials and methods

2.1. Cell lines

BL60 is a group I Burkitt's lymphoma cell line which bears a surface phenotype characteristic of germinal centre B lymphoblasts ($CD10^+$ $CD20^+$ $CD23^-$ $CD39^-$ $CD77^+$) and only expresses the Epstein Barr Virus protein EBNA-1 [10]. The B lymphoma cell line B104 [11,12] was established from peripheral blood mononuclear cells of a child bearing a malignant lymphoma at the leukemic stage. This cell line was kindly provided by Dr. M. Mayumi (Kyoto, Japan). The cell line VAL was derived from a patient with acute lymphoblastic leukaemia [10]. It bears a double translocation t(8;14;18)(q24;q32;q21). This cell line was kindly provided by C. Bastard (CRTS, Bois Guillaume, France). The murine B lymphoma cell line WEHI-231 [12] and the human myelomonocytic cell line HL60 were obtained from the American Type Culture Collection (ATCC Rockville, MD). Thymocytes were obtained from the thymuses of BALB/c mice bred in our laboratory. Cells were cultured at 37°C in a moist atmosphere of 5% CO_2, in complete medium made of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The medium for B104 and WEHI-231 cell lines was supplemented with 2-mercaptoethanol at a concentration of 5×10^{-5} M.

2.2. Induction of apoptosis

Cells were cultivated to 0.2×10^6 cells/ml for cell lines and 10^6 cells/ml for mouse thymocytes in complete medium with etoposide (10 μg/ml; kindly provided by Sandoz Pharmaceutical, Basel, Switzerland), ionomycin 1 μg/ml (obtained from Calbiochem-Behring Corp., LaJolla, CA). B104 cells were also treated with anti-human IgM (μ chain specific) (20 μg/ml; from Bio-Rad Laboratories, Hercules, CA) and WEHI-231 cells were treated with the $F(ab')_2$ fragments of anti-mouse IgM rabbit antibodies (μ chain specific) (1 μg/ml; from Cappel, Durham, NC).

2.3. Evaluation of cell death

The morphological features of cells following various treatments were observed using transmission electron microscopy. After two washings with phosphate-buffered saline, cells were fixed in 2% osmium tetroxide in 0.1 mol/l cacodylate buffer, pH 7.4, dehydrated and embedded in Epon. Thin sections were cut and following lead citrate and uranyl acetate contrasting, were observed in a Jeol 100CS electron microscope. Cells were also analyzed by fluorescence microscopy, after staining with Hoechst 33342 (Sigma Chemical Co., St. Louis, MO) at 10 μg/ml following previously described methods [3], under an epifluorescence Zeiss microscope (Zeiss, Oberkochen, Germany) using a ×100, 1.25 numerical aperture, oil immersion objective. Nuclear fragmentation and/or marked condensation of the chromatin with reduction of nuclear size were considered as typical features of apoptotic cells [3,13,14]. Based on these criteria, results were expressed as percentage of apoptotic cells.

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2.4. Measurement of cell DNA content by flow cytometry

For DNA content analysis, cells washed in phosphate buffered saline were stained with propidium iodide (50 μg/ml in 0.1% Triton X-100, EDTA 0.1 mM, RNase 50 μg/ml) after fixation with 70% ethanol overnight at 4°C in darkness [15]. Cell suspensions were then analyzed with a Facstar plus flow cytometer (Becton Dickinson, Pont de Claix, France) using an argon laser ($\lambda_{Ex.Max.}$ 540 nm, $\lambda_{Em.Max.}$ 620 nm). Histograms of DNA content were drawn using the Lysis II program and relative percentages of cells in the G_0/G_1 or S/G_{2M} phases of cell cycle and cells with a lower DNA content than those in G_0/G_1, referred to as the 'Sub-G1' population typical of apoptotic cells [3] were determined after exclusion of debris by electronic gating of the cytogram drawn from light diffraction at forward and right angle scatters as previously described [16].

2.5. Agarose gel electrophoresis

DNA preparations were obtained following a procedure previously described [5]. Briefly, 5×10^6 cells were lysed in a buffer containing 10 mM EDTA, 100 mM NaCl, 0.5% (w/v) SDS, 100 mM Tris-HCl, pH 7.4, and 0.1 mg/ml proteinase K (Boehringer, Mannheim, Germany). The DNA was extracted twice with phenol/chloroform and twice with a chloroform/isoamyl alcohol mixture. The aqueous phase was precipitated with two volumes of ethanol. Unfragmented DNA was discarded, and 3 M sodium acetate (to one-tenth volume) was added to the supernatant followed by incubation at −20°C overnight. Fragmented DNA was centrifuged, dried under vacuum, and resuspended in 100 μl of RNase (1 mg/ml) buffer containing 10 mM Tris and 1 mM EDTA, pH 7.5. The samples were diluted in loading buffer and loaded on a 2% agarose gel containing 0.1 μg/ml ethidium bromide. DNA fragments were visualized using ultraviolet light.

2.6. Clamped homogeneous electric field

CHEF was carried out according to the method of Chu et al. [17]. Briefly, electrophoresis was carried out using a horizontal gel chamber, a model 200/2.0 power supply and Pulsewave 760 switcher (Bio-Rad Laboratories). The gels were run at 200 V in 225 mM Tris-Base, 225 mM boric acid and 5 mM EDTA with a ramping rate changing from $T_1 = 12$ s to $T_2 = 30$ s for 20 h with a forward-to-backward ratio of 1 at 14°C using 0.8% electrophoresis grade agarose. After electrophoresis, the gel was stained with ethidium bromide (10 mg/ml; 15 min) and then destained in running buffer (1 h). DNA fragments were visualized using ultraviolet light. Molecular weight markers (48.5–1018.5 kpb) were purchased from Biolabs (Beverly, MA).

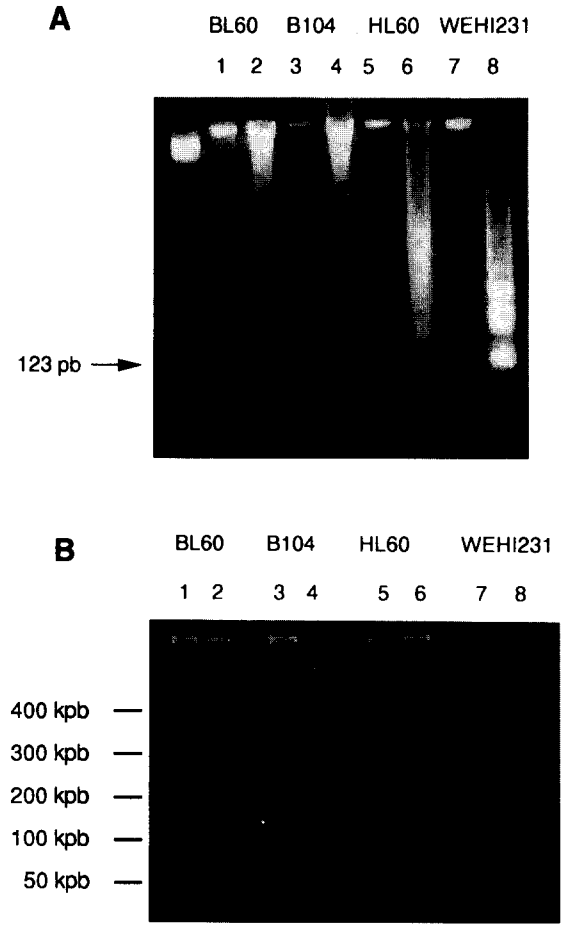

Fig. 1. DNA fragmentation after treatment of various cell lines with etoposide. Cells (0.2×10^6 cells/ml) were treated with (lanes 2, 4, 6, 8) or without (lanes 1, 3, 5, 7) etoposide 10 μg/ml during 20 h. DNA fragmentation was evaluated on agarose gel electrophoresis (A) or on CHEF pulsed field electrophoresis gel (B).

3. Results

3.1. DNA fragments size after apoptosis depends on cell types

Etoposide is an inhibitor of topoisomerase II which induces apoptosis in various cell types [3,18]. We have tested this inhibitor on four human cell lines, HL60, BL60, B104 and VAL, on

Table 1
Comparison of various apoptosis parameters

Cell type	Apoptosis inducers	'sub G1' population	Observed DNA fragments sizes		Nuclear morphology after Hoechst staining	
			Small	Large	Condensation	Fragmentation
Thymocytes	None	+	+	−	nd	nd
	Etoposide	+	+	−	4%	96%
	Ionomycin	+	+	−	nd	nd
WEHI 231	Etoposide	+	+	−	3%	97%
	Ionomycin	+	+	−	nd	nd
	Anti-IgM	+	+	−	1%	99%
HL60	Etoposide	+	+	−	1%	99%
B104	Etoposide	−	−	+	22%	78%
	Ionomycin	−	−	+	25%	75%
	Anti-IgM	−	−	+	nd	nd
BL60	Etoposide	−	−	+	23%	77%
	Ionomycin	−	−	+	22%	78%
VAL	Etoposide	±	±	±	nd	nd

'sub G1' population: + = presence of sub G1 population after flow cytometry analysis of DNA content. *Small DNA fragments:* + = presence of DNA fragmentation (180–200 pb) visible on agarose gel electrophoresis. *Large DNA fragments:* + = presence of DNA fragmentation (100–150 kbp) visible on CHEF pulsed field electrophoresis gel. nd = not determined.

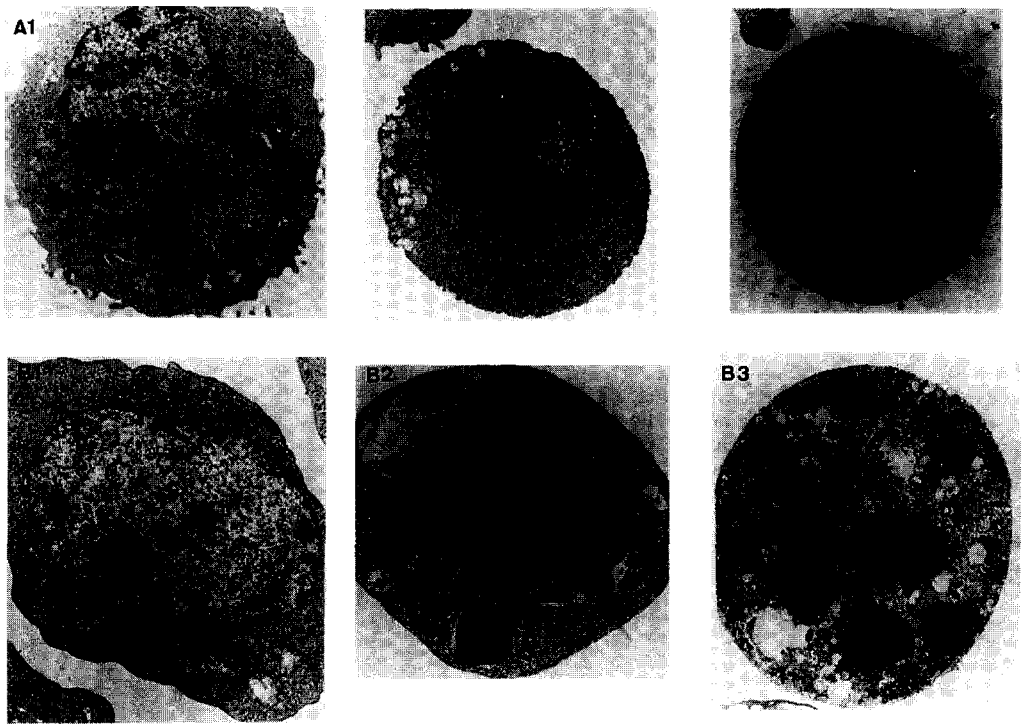

Fig. 2. Ultrastructural morphology of etoposide-treated cells. BL60 (A) or HL60 (B) were treated with etoposide 10 μg/ml during 16 h and morphology was studied in electron microscopy. Normal cells (A1 and B1) and cells with apoptotic morphology, condensed nuclei (A2 and B2) or fragmented nuclei (A3 and B3) are presented here.

the murine B cell line WEHI-231 and on mouse thymocytes. Morphological studies of these cells after treatment with etoposide at 10 μg/ml for 6 h showed typical features of apoptosis. DNA fragmentation was evaluated after treatment with etoposide for 20 h. The internucleosomal fragmentation of nuclear DNA with typical ladder appearance in agarose gel electrophoresis was observed in HL60 and WEHI-231 cells but not in BL60 and B104 cells (Fig. 1A). Similar fragmentation was documented in mouse thymocytes undergoing apoptosis (Table 1). In contrast, large DNA fragments of 100–150 kb were observed in BL60 and B104 cells but not in HL60 and WEHI-231 cells treated with etoposide (Fig. 1B). VAL cells showed an intermediate profile with both small and large DNA fragments (Table 1).

WEHI-231, B104, BL60 and mouse thymocytes were treated with the calcium ionophore ionomycin (1 μg/ml) which induces apoptosis [19–21]. Small DNA fragments were observed in WEHI-231 cells and thymocytes but only large DNA fragments were present in BL60 and B104 cells undergoing apoptosis. Similarly, B104 cells treated with anti-human IgM generated large DNA fragments whereas WEHI-231 treated with anti-mouse IgM antibodies contained only small DNA fragments. These results suggest that the type of DNA fragmentation in various cell lines is an intrinsic characteristic of the cell lines used rather than a property of the agent used to trigger apoptosis.

3.2. *Absence of nuclear fragmentation is not uncommon in apoptotic cells displaying large DNA fragments*

The morphological characteristics of apoptotic cells in electron microscopy were heterogeneous within each cell type, with condensation of the chromatin in tight apposition to the nuclear envelope, condensation of the whole nucleus and fragmentation of the nucleus. These three ultrastructural aspects were observed in various proportions in the five cell types induced to apoptosis by etoposide (Fig. 2). To evaluate the relative occurrence of each morphological feature of apoptosis, cells treated with etoposide were examined by UV microscopy after staining with Hoechst 33342 and apoptotic cells were characterized by either condensation or fragmentation of their nuclei. The percentages of cells with condensed but non-fragmented nuclei was greater in the BL60 and B104 cell lines (large DNA fragmentation) than in the HL60 and WEHI-231 cell lines (small DNA fragmentation) (Table 1).

3.3. *DNA content decreases only in cells producing small DNA fragments*

We measured cellular DNA content by propidium iodide staining after permeabilisation with 70% ethanol and 0.1% Triton X-100. The percentage of hypodiploid cells was determined by flow cytometry and the percentage of apoptotic cells by Hoechst staining. As shown in Fig. 3, despite comparable percentage of apoptotic cells induced by etoposide in each of the four cell lines, no significant 'subG1' population was observed in BL60 and B104 whereas this population was markedly increased in HL60 and WEHI 231 (Fig. 3) and in thymocytes (data not shown) treated with etoposide.

4. Discussion

Agarose gel electrophoresis of DNA showing a typical 'laddering' aspect is generally considered as the reference method

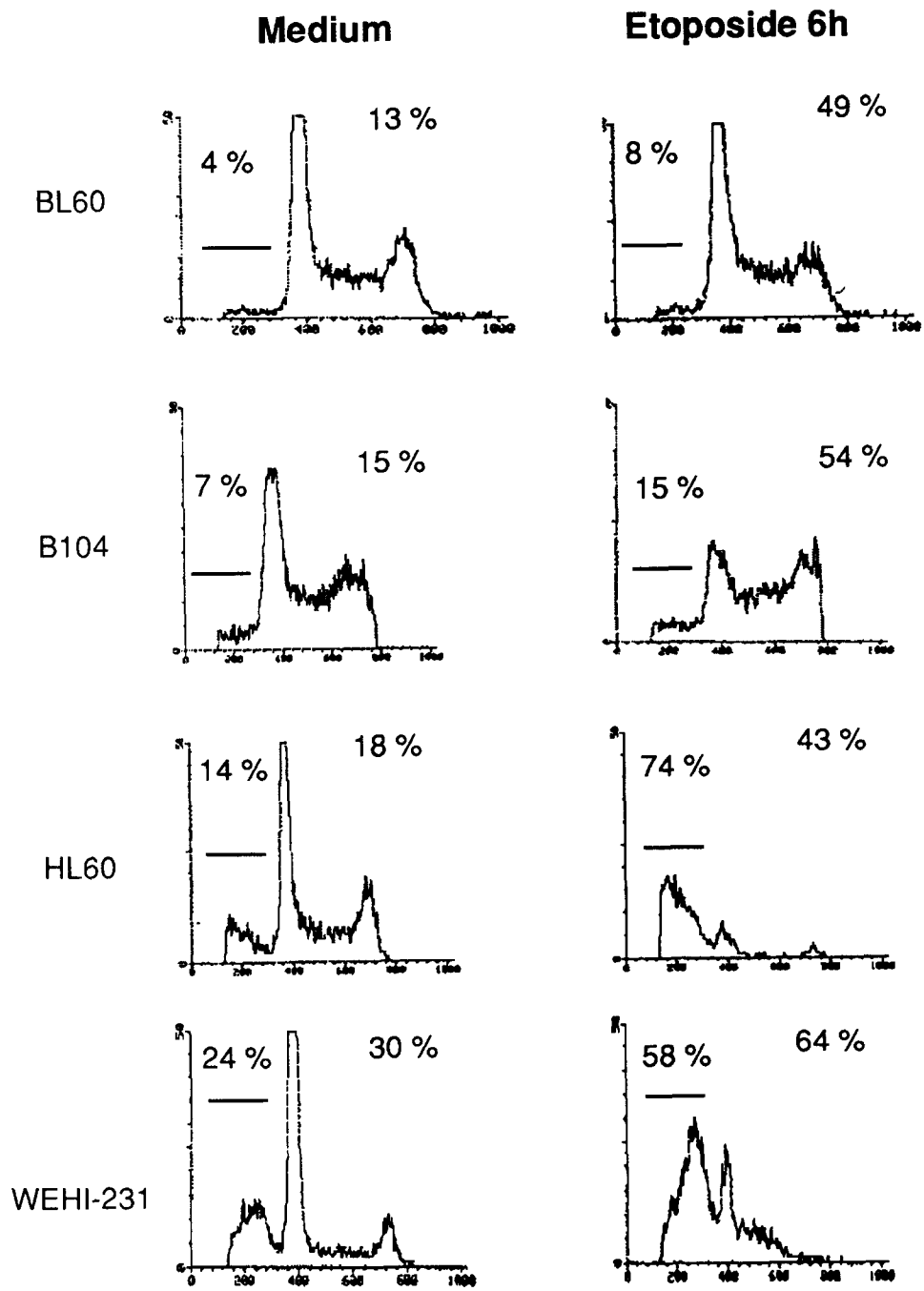

Fig. 3. Occurrence of a 'subG1' population in cells treated by etoposide. Cells (0.2×10^6 cells/ml) were treated with (right panel) or without (left panel) etoposide 10 μg/ml for 6 h. DNA content was analyzed by flow cytometry after permeabilisation with 70% ethanol and 0.1% Triton X-100 and staining with propidium iodide. Left values represent the percentage of cells in the 'subG1' population and right values represent the percentage of cells with apoptotic morphology evaluated by UV microscopy after Hoechst 33342 staining.

for demonstrating apoptosis [5–8]. More recently, Darzynkiewicz et al. [3] reported that the occurrence of a cell population with a decrease of DNA content (hypodiploid or 'subG1' population) was another characteristic of apoptotic cells. Most studies in support of these markers of apoptosis have used mouse thymocytes or the human myelomonocytic cell line HL60. The recent description of apoptosis with another type of DNA fragmentation (50–300 kb) in epithelial cell lines [9], in thymocytes [22] and in a monocytic cell line [23] led us to investigate whether the latter type of DNA fragmentation could be observed in other cell lines and whether or not it was associated with a decrease of cell DNA content.

Etoposide is an inhibitor of topoisomerase II which was reported to induce apoptosis in various cell lines [3,18]. We have tested this inhibitor on four human cell lines, HL60, BL60, B104 and VAL, on the murine cell line WEHI-231 and on

mouse thymocytes. Our results show that most of the cell types studied produced either small or large DNA fragments. However, in the human B cell line VAL the two types of fragmentation were observed. It could be hypothesized that generation of large DNA fragments represents an early alteration eventually leading to further fragmentation as it has been described on murine thymocytes treated by dexamethasone [22]. Only electrophoresis of cells collected after 20 h of etoposide treatment, which correspond to late time points in the kinetics of etoposide-induced apoptosis are presented here. Actually DNA electrophoresis was performed at different time intervals (6, 10, 16, 20 h) after addition of etoposide but we did not observe a shift from large to small DNA fragments (data not shown). Although very early formation of large DNA fragments in cells which will eventually generate small DNA fragments cannot be ruled out, the type of DNA fragmentation seems to represent an intrinsic property of each cell type. An additional support to this view comes from experiments in which apoptosis was induced by the calcium ionophore ionomycin or by cross-linking of surface immunoglobulins. In these cases the type of fragmentation was identical to that induced by etoposide. Interestingly, Ishigami and coworkers reported that B104 cells treated with anti-IgM antibodies undergo cell death with apoptotic morphology in electron microscopy in the absence of small DNA fragments [12]. Our results show that B104 cell death involves apoptosis resulting in the generation of large DNA fragments.

It is remarkable that the two types of DNA fragmentation were associated with similar ultrastructural features although the relative proportion of cells with condensed non-fragmented nuclei was slightly increased in cells undergoing apoptosis with large DNA fragments.

Measurement of cell DNA content by flow cytometry clearly demonstrated that only apoptotic cells with small DNA fragments had decreased DNA content corresponding to the 'subG1' population. Darzynkiewicz et al. [3] reported that decrease of cell DNA could be attributed to the migration of small DNA fragments through the cytoplasm, followed by their exclusion from the cell. We may assume that, due to their size, large DNA fragments are hampered in their migration from the nucleus to the extracellular space.

Considering our results with B104 and BL60 cell lines, the occurrence of small DNA fragments in agarose gel electrophoresis, and that of a decreased DNA content with a 'subG1' cell population in flow cytometry, should no longer be considered as reference criteria to determine cell apoptosis. On the other hand, both ultrastructural features and morphology of the nucleus after staining with Hoechst 33342 appear as reliable criteria of apoptosis, regardless of the size of DNA fragments viewed after apoptosis.

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