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Image analysis of DNA fragmentation and loss in V79 cells under apoptosis

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Abstract

Nuclear image analysis of Feulgen-stained V79 fibroblasts after three days in culture was used to discriminate apoptotic cells and cells suspected to be undergoing apoptosis from control cells based on parameters such as the Feulgen-DNA content, degree of chromatin condensation and nuclear areas, in association with visual morphology. The fibroblasts were initially plated at a density of 10^5 cells/ml and incubated under optimal culture conditions without subculturing. Following confluency, the cells underwent contact inhibition apoptosis. Image analysis revealed three nuclear phenotypes which were defined in terms of their morphological characteristics and levels of chromatin condensation. A decrease in the amount of Feulgen-DNA was detected in apoptotic cells and in cells suspected of undergoing apoptosis. This decrease was assumed to indicate DNA loss. Image analysis procedures may therefore provide a useful tool for discriminating cells in the early stages of apoptosis.

INTRODUCTION

Cells in advanced stages of apoptosis exhibit morphological changes characterized by nuclear and cytoplasmic condensation and cell fragmentation into membrane-bound apoptotic bodies (Wyllie *et al.*, 1981). These morphological changes can be observed by light microscopy and are correlated initially with large (50 to 300 kb) and, subsequently, very small (oligonucleosomes, 180-200 bp) chromosomal DNA fragments (Wyllie *et al.*, 1981).

Internucleosomal DNA digestion is one of the hallmarks of apoptosis and is frequently used as the sole criterion for its detection (Arends *et al.*, 1990; Cohen *et al.*, 1991; Raff, 1992). However, no clear evidence has been presented that DNA degradation plays a primary and causative role in apoptotic cell death (Schulze-Osthoff *et al.*, 1994). Indeed, other morphological features of apoptosis occur in the absence of detectable DNA fragmentation or a decrease in DNA content (Cohen *et al.*, 1991; Falcieri *et al.*, 1993; Oberhamer *et al.*, 1993; Fournel *et al.*, 1995; Zamai *et al.*, 1996).

The existence of a variety of apoptosis inducers and of variable expression according to the cell type considered has led to a reconsideration of the morphological criteria for the detection of apoptosis. Even in studies using *in situ* labeling of DNA strand breaks as a marker, there is a heavy reliance on morphological features for the identification of apoptotic nuclei.

When associated with image analysis procedures, the Feulgen reaction has proven useful for evaluating changes in chromatin texture in different cell types under various experimental conditions (Mello, 1989, 1999; Mello and Russo, 1990; Mello *et al.*, 1995; Vidal *et al.*, 1998). Feulgen-stained preparations have also been used to quantify apoptotic and mitotic indices (Camby *et al.*, 1995) and to establish apoptosis-cell cycle relationships (Wallet *et al.*, 1996).

Image analysis of Feulgen-stained nuclei should be capable of revealing changes in chromatin supraorganization as well as in DNA amounts during apoptosis. In addition, compared to the TUNEL assay, such analysis should allow the detection of the early stages of apoptosis by associating slight changes in nuclear morphology with a decrease in DNA content. In this study, DNA loss and changes in chromatin texture in hamster V79 fibroblasts were evaluated by image analysis of Feulgen-stained preparations and then examined for their correlation with the morphological features of apoptosis. In long-term cultures, these cells show classic characteristics of apoptosis (Maria and Vidal, 1996).

MATERIAL AND METHODS

Cells

Chinese hamster V79 lung fibroblasts (clone M8) at passage 34 were supplied by Dr. Marcela Haun (Department of Biochemistry, UNICAMP). The cells were plated at a density of 10^5/ml on 90-mm Petri dishes containing six 18 x 18-mm coverslips and 10 ml of culture medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU penicillin/ml and 100 IU streptomycin/ml. The preparations were incubated at 37°C in a humidified chamber with 5% CO_2 in an atmosphere of air and the medium was changed daily. The cells were used after 3 days of incubation, to avoid the problem of detached confluent cells, which occurs in 6-day cultures.

Cell fixation and staining

The cells were fixed in absolute ethanol-acetic acid (3:1, v/v) mixture for 1 min, rinsed in 70% ethanol for 5

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min and air-dried at room temperature. They were then subjected to the Feulgen reaction, the acid hydrolysis step of which was carried out in 4 M HCl at 25°C for 90 min. The stained preparations were mounted in Canada balsam (n_D = 1.54).

Image analysis

Video image analysis cytometry was carried out according to Vidal (1997), using a Global Lab Image software (Data Translation, Inc. System, Marlboro, MA, USA) coupled to a Zeiss photomicroscope equipped with a Pol-Neofluar 25/0.60 objective, 1.25 optovar, 1.4 condenser, λ = 546 nm, and a 12-v 60-w lamp connected to a stabilizer. The images to be processed were transmitted from the microscope to the computer by a JVC monochromatic CCD video camera. The conversion of pixels to µm was done using a micrometer slide as standard. Nuclear areas (S), optical densities (absorbances, OD), and integrated optical densities (IOD or, in this case, Feulgen-DNA values) were chosen from several descriptors provided by the software based on the information required by this study. The conversion of gray levels to absorbances or transmittances was done using the Minitab program.

TUNEL assay

Cells fixed in absolute ethanol-acetic acid (3:1, v/v) were subjected to the TUNEL assay (*In situ* Cell Death detection kit, Böhringer Mannheim, Germany) to detect apoptosis according to the manufacturer's instructions.

Statistical analysis

The experiments were performed in triplicate and repeated at least three times. Statistical analysis involved analysis of variance (ANOVA) and the Mann-Whitney non-parametric test.

RESULTS

The cells grew exponentially and became confluent after 3 days in culture. Apoptotic cells appeared after confluency, without subculturing. Three interphase phenotypes differing in their chromatin packing states and intranuclear distribution occurred in the 3-day cultures. The most frequent phenotype (I) was characterized by a few small chromatin granules in a homogeneously distributed, delicate chromatin background and was considered the control (Figure 1a). Another phenotype (II), designated as suspected apoptosis, exhibited larger areas of condensed chromatin and a reduced nuclear area compared to type I nuclei (Figure 1b; Table I). In the third phenotype (III) the nuclei showed chromatin condensation typical of apoptosis (Figure 1c,d), with clumps of condensed chromatin distributed at the nuclear periphery. Entire III nuclei frequently appeared condensed into clusters of round corpuscles (Figure 1c). The incidence

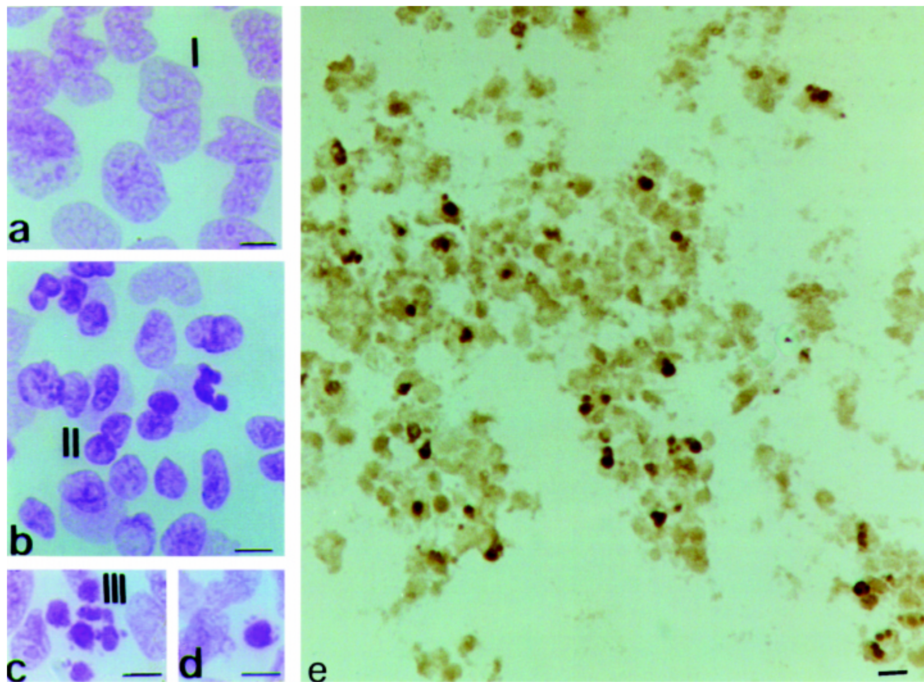

Figure 1 - Nuclear aspect of V79 fibroblasts after three days in culture. Different types of chromatin condensation are present in Feulgen-stained nuclei (a-d): I (control, a), II (suspected apoptosis, b), and III (apoptosis, c,d). The TUNEL assay confirmed occurrence of apoptosis in the cell population (e). Bar = 10 µm.

Table I - Video nuclear image parameters for Feulgen-stained V79 fibroblasts.

Nuclear phenotypes	N	OD			IOD			S (μm²)		
		$\bar{X}$	SD	η	$\bar{X}$	SD	η	$\bar{X}$	SD	η
I	452	0.20	0.05	0.20	26.20	12.78	23.30	132.78	56.25	118.98
II	81	0.24	0.06	0.22	23.01	17.65	14.60	90.38	50.69	70.60
III	52	0.30	0.09	0.29	16.58	7.57	15.37	63.92	44.42	46.95

IOD, Integrated absorbances; N, number of measurements; OD, absorbances; S, nuclear absorbing area; SD, standard deviation; $\bar{X}$, arithmetic mean; η, median.

of apoptosis in this cell population was confirmed by the TUNEL assay (Figure 1e).

The nuclear images studied with the video image analyzer were chosen based on their morphological characteristics. The number of measurements performed varied with the frequency of each nuclear phenotype. The apoptotic nuclei chosen for measurements showed no signs of nuclear envelope disruption.

The OD values of the type II nuclei were greater than those of type I nuclei but smaller than those of apoptotic nuclei. In contrast, the Feulgen-DNA content and the nuclear absorbing area of type II nuclei were smaller than those of type I nuclei, but greater than those of apoptotic nuclei (Tables I and II).

DISCUSSION

The Feulgen reaction was capable of highlighting nuclear images typical of apoptosis and nuclei suspected of being committed to cell death by apoptosis. Image analysis confirmed the visual discrimination, by distinguishing nuclei at two different stages of apoptosis.

The decrease in the amount of nuclear Feulgen-DNA with the apoptosis may have resulted from an intrinsic loss of fragmented DNA or changes in its physicochemical stability to the Feulgen acid hydrolysis. The first hypothesis appears to be the most reasonable since DNA loss has been reported for apoptotic nuclei following procedures, such as flow cytometry, which do not involve acid hydrolysis (Ferlini *et al.*, 1996). DNA loss in V79 cells during apoptosis probably involves the action of endonucleases.

Table II - Comparison of nuclear image analysis parameters in V79 fibroblasts.

Descriptors	Compared phenotypes	Mann-Whitney (P)	ANOVA (F)
Absorbances (= OD)	I, II, III		94.93**
	I vs. II		59.05**
	II vs. III		18.79**
Feulgen-DNA content (= IOD)	I vs. II	0.0000**	
	II vs. III	0.0246*	
	I vs. III	0.0000**	
Nuclear absorbing area	I vs. II; II vs. III; I vs. III	0.0000**	

*Significant ($P_{0.05}$ level); **highly significant ($P_{0.01}$ level).

The idea that the presence of fragmented DNA arising from endonuclease action reflects the fact that apoptotic nuclear DNA is more sensitive to acid hydrolysis during the Feulgen reaction does not seem to apply here. Indeed, cytochemical studies have shown that the fragmented DNA forms in apoptotic nuclei of V79 and BHK21 fibroblasts complex with histones and concanavalin A-reactive proteins in the same way as DNA from non-apoptotic nuclei (Vidal, B.C. and Maria, S.S., unpublished observations).

Flow cytometry suggests that alterations in chromatin texture in early apoptosis lead to changes in DNA stainability (Ferlini *et al.*, 1996). However, the usefulness of flow cytometry is limited by its inability to preserve cell topography, the need for fluorescent labels which vary in affinity for DNA, and the need for a large cell sample (Wallet *et al.*, 1996). In contrast, the Feulgen reaction, besides being highly stoichiometric, is capable of showing the morphological characteristics of apoptotic chromatin *in situ* (Camby *et al.*, 1995; Mello, 1999).

The gradual increase in OD values concomitant with a decrease in nuclear areas favors the idea of enhanced chromatin condensation in nuclei supposedly at an early stage of apoptosis (type II nuclei). The type I nuclei which exhibited OD and nuclear area characteristics similar to those of type II nuclei may represent intermediate stages of chromatin supraorganization prior to becoming type II nuclei.

The apoptosis described here for V79 fibroblasts was induced solely by advancing cell growth time in culture and maintenance of cell-cell interactions (Dini *et al.*, 1996). In the presence of drugs and other agents, the nuclear morphology and chromatin texture of V79 apoptotic cells may well differ from those described here.

In conclusion, the phenotypical variation in the apoptotic process seen in Feulgen-stained preparations was confirmed by image analysis data. Morphologically suspect cell nuclei may be shown to be early apoptotic cell nuclei, by associating visual microscopic observations

with image analysis descriptors which demonstrate a decrease in DNA content.

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RESUMO

Análise de imagem de núcleos de fibroblastos V79 após três dias em cultura foi realizada em preparados submetidos à reação de Feulgen para discriminar células suspeitas de estarem em fases precoces da apoptose daquelas comprovadamente apoptóticas. Parâmetros tais como conteúdo de Feulgen-DNA, grau de condensação cromatínica e área nuclear foram estudados em associação com a morfologia estabelecida em termos visuais. Os fibroblastos foram inicialmente plaqueados numa densidade de 10^5 células/ml e encubados sob condições ótimas de cultura sem subcultura. Com a confluência, as células sofreram apoptose pela inibição por contato. A análise de imagem revelou três fenótipos nucleares definidos quanto a características morfológicas e níveis de condensação cromatínica. Foi detectado decréscimo no conteúdo de Feulgen-DNA das células apoptóticas mas também nas células suspeitas de apoptose. Admite-se que este decréscimo indique perda de DNA. A análise de imagem pode ser, portanto, uma ferramenta útil na discriminação de células mesmo em fases precoces da apoptose.

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