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DIFFERENT APPROACHES TO THE STUDY OF APOPTOSIS

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Abstract

The morphological features of cell undergoing programmed cell death is well known and has been widely described in a number of experimental models with a variety of apoptotic triggering agents. Despite the similar cell behaviour, underlying molecular events seem variable and only partially understood. A multiple approach appears crucial to better clarify the phenomenon. The first technique, DNA gel electrophoresis, allows the identification of fragmented DNA and has been long considered the hallmark of apoptosis. Different patterns of DNA cleavage, which can be identified by conventional or "pulsed-field gel" electrophoresis, are presented and discussed. "In situ" labelling methods are also described both with terminal deoxynucleotidyl transferase and DNA polymerase I, aimed at the study of the distribution of DNA cleavage areas. Flow cytometry is also proposed and different technical approaches, based on different laser utilizations, are discussed. Ultrastructural analysis, allowing the study of apoptotic cell details, is finally considered.

Key Words: Apoptosis, DNA electrophoresis, "in situ" DNA labelling, flow cytometry, electron microscopy, freeze-fracture.

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Introduction

Apoptosis, widely described "*in vivo*" and "*in vitro*," appears as a crucial process in cell deletion during development, in adult tissue homeostasis and in antineoplastic chemotherapy. The light and electron microscopy features of programmed cell death consist of a very particular nuclear behaviour involving chromatin progressive margination and compacting, with the final appearance of numerous, homogeneously dense, micronuclei, scattered throughout the cytoplasm (Wyllie *et al.*, 1980; Arends and Wyllie, 1991; Falcieri *et al.*, 1994a). In contrast with necrotic death, plasma membrane and organelles appear well preserved for long periods (Falcieri *et al.*, 1994b).

In the final stages, the apoptotic cell splits in a number of "apoptotic bodies" which undergo secondary necrosis or phagocytosis by neighbouring cells (Dini, 1996). Morphological observations suggest a very similar behaviour of chromatin and other nuclear domains in all apoptotic models despite the strong differences among target cell lineages and triggering agents (Falcieri *et al.*, 1994c). A common step is also present, accounting for the rearrangement of cell architecture, but presumably preceded by different and specific metabolic events, still partially unknown, correlated to the various cell conditions (Collins *et al.*, 1992; Falcieri *et al.*, 1993; Fady *et al.*, 1994). DNA oligonucleosomic fragmentation, induced by the activation of a Ca^{2+} dependent endonuclease has long been considered a hallmark for apoptosis identification (Arends *et al.*, 1990). When agarose gel electrophoresis is performed, a characteristic ladder appears, indicating the presence of small size DNA fragments (Bøe *et al.*, 1991). Recently, apoptotic features have been described, in which the typical chromatin changes were not supported by the previously described DNA fragmentation, so involving different and still unexplored mechanisms (Oberhammer *et al.*, 1993a,b).

A multiple technical approach appears very useful for clarifying the events involved in programmed cell death and is discussed in this paper.

Abbreviations

TdT	Terminal deoxynucleotidyl Transferase
Pol I	DNA Polymerase I
dNTP	deoxy-Nucleotidyl-Triphosphate
dUTP	deoxy-Uridin-Triphosphate
DIG	digoxigenine
FITC	fluoresceine isotiocynate
PI	propidium iodide
NT	nick translation

Materials and Methods

Apoptotic models

Thymocytes were obtained from two-week-old BALB/c mice (Charles River, Milano, Italy) and incubated for 24 hours in RPMI 1640, 10% fetal calf serum (FCS), 10 mM Hepes buffer, 0.1 M dexamethasone (Sigma, St. Louis, MO, USA) at 37°C, as previously described (Vitale *et al.*, 1993).

HL60 human leukaemia cells were used in the log phase of growth. Apoptosis was induced by 0.15 M camptothecin (Sigma) for 6 hours at 37°C, 5% CO_2 (Kaufmann, 1989; Darzynkiewicz *et al.*, 1992), or by addition of 10^{-1} M methotrexate.

Human lymphocytic leukaemia **MOLT-4 cells** (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI 1640 and induced to apoptosis by the addition of 20 nM staurosporine for 24 hours (Boehringer, Mannheim, Germany), as previously reported (Falcieri *et al.*, 1993).

P815 murine mastocytoma cells were routinely cultured and exposed to mild hyperthermia to induce apoptosis (Liepins, 1989; Collins *et al.*, 1992).

Untreated cells were used as controls in all the different models and for each of the technique used.

DNA gel electrophoresis

DNA gel electrophoresis was performed after DNA cell extraction (Bøe *et al.*, 1991). Purified DNA was run on an agarose gel and compared with commercial weight markers or standard fragmented DNA, and oligonucleosomic DNA fragments can so be identified.

Using the "pulsed-field gel" electrophoresis (PFGE) technique, double stranded DNAs, in the range of 1^{-10} Mbp, can be separated. It consists in the use of several and differently angled electric fields which are applied in pulses. Molecules are so stretched towards the field direction, successively relaxed, and newly stretched in another direction. Longer molecules relax less than the shorter ones, and so take longer to start moving in the new direction, thus, acquiring a progressively different spatial location in the gel.

In brief, the method entails 5 x 10^7 cells/ml re-suspended in PBS 1x and embedded in 1% low melting agarose. The plugs are digested for 48 hours in a lytical buffer containing 50 g/ml proteinase K, 25 mM ethylenediaminetetraacetic acid (EDTA) and 1% sarkosylate. They are then washed with 0.05 M EDTA and stored at 4°C. Before run, the plugs are placed into the wells of 1% agarose gel and cooled at 14°C for 30 minutes in the running buffer (0.5x TAE). The migration of *S. cerevisiae* DNA size standard and of DNA from staurosporine treated cells are performed at 6 V/cm voltage, 14°C, 24 hour run times, a switch time from 45 to 90 seconds, 120° angle and linear ramping. The gel is finally stained with 5 g/ml ethidium bromide for 30 minutes (Sambrook *et al.*, 1989; Ito *et al.*, 1993; Oberhammer *et al.*, 1993b; Walker *et al.*, 1995).

In situ detection of DNA breaks

Chromatin alterations in apoptotic cells can be demonstrated "*in situ*" by means of two techniques able to identify the presence of DNA breaks, labelled using TdT or Pol I, respectively. In this approach, treated and control cells are fixed with 3:1 methanol-acetic acid, dropped onto slides and treated with TdT or Pol I, which induce incorporation of a labelled dNTP, usually biotin-dUTP or DIG-dUTP. Incorporated dNTPs are detected by means of specific FITC conjugated antibodies and the reaction is observed through a conventional fluorescence microscope or a confocal scanning laser microscope after counter-staining with PI (Telford *et al.*, 1992).

The TdT enzyme works on a template represented by a single strand DNA with a 3' OH terminus or, in presence of the $CoCl_2$ in the reaction buffer, on a blunt ended, double stranded DNA (Fig. 1). The reaction consists of the addition of labelled dNTP to 3' OH termini of DNA molecules, and it can be considered as able to show DNA breaks occurring on both strands.

On the other hand, the Pol I recognizes as template a double stranded DNA with the presence of a nick which serves as a primer for a NT reaction, in which the 5'-3' exonuclease activity of Pol I removes the dNTPs originally present in one strand and the 5'-3' polymerase activity allows the incorporation of the labelled dUTP (Fig. 2). However, experiments performed by means of "*in situ*" digestion with restriction enzymes suggest that, when the template is represented by chromatin, instead of nicked DNA, Pol I is able to work on blunt ended or sticky ended double stranded DNA as well (Adolph and Hameister, 1985; De La Torre *et al.*, 1992; Falcieri *et al.*, 1994a).
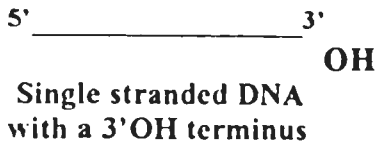
Flow cytometry

FACS (fluorescent activated cell sorter) is most commonly utilized to separate and score differently fluorescent cells.
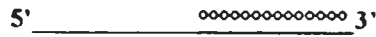
Terminal-deoxynucleotidyl-Transferase   1
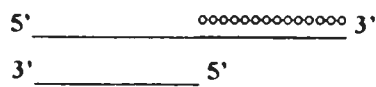
Template: ## Reaction:

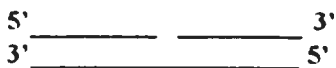5'_____3'   5'_____ooooooooooooo 3'
                    OH
Single stranded DNA
with a 3'OH terminus

or (in presence of Co++)

5'_____3'   5'_____ooooooooooooo 3'
3'_____5'   3'_____ 5'

Blunt ended, double stranded DNA   **Addition of d-NTPs to the 3'OH termini**

                                                                   2
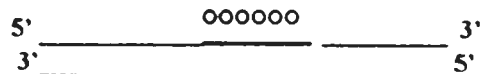
DNA Polymerase I

Template: ## Reaction:

5'_____ _____3'   5'_____oooooo_____3'
3'_____5'   3'_____5'

Double stranded DNA with presence   **Incorporation of d-NTPs by means of**
of a nick which serves as a primer   **the 5'-3' exonuclease and polymerase**
for DNA synthesis   **activities**

Figures 1 and 2. Schemes of TdT "*in situ*" labelling (**Fig. 1**) and of Pol I "*in situ*" NT (**Fig. 2**) of cleaved DNA.

For the detection of apoptosis, it can be used in different ways. A first approach is based on the presence of a subdiploid peak corresponding to the apoptotic cell population after ethanol fixation and incubation with a DNA specific fluorescent dye. The most common stain is PI, a stoichiometric DNA intercalating agent, which generates an hypodiploid peak that identifies apoptotic cells. Ethanol permeabilized apoptotic cells, in fact, loose part of the fragmented DNA (Darzynkiewicz *et al.*, 1992).

A second approach consists in the identification of apoptotic cells by means of the side scatter parameter. Side scatter is generated by the combination of the refracted and reflected components of the light scattered by the cell, and is therefore a function of the "inner cell complexity." Since apoptosis is a process that takes place mainly in the nucleus while the cytoplasm appears preserved for long, the higher the nuclear/cytoplasmic ratio of the cell, the better the side scatter parameter discriminates between normal and apoptotic cells, particularly in their early stages (Zamai *et al.*, 1993; Zauli *et al.*, 1994).
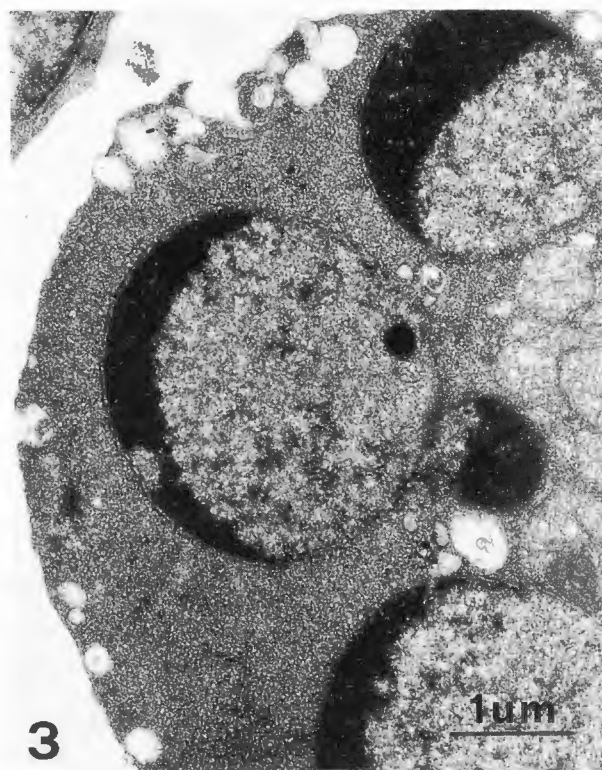
Figure 3. Molt-4 cell in presence of staurosporine: the chromatin margination, characteristic of apoptosis, appears.

A third approach utilizes PI uptake in unfixed cells. Even if ultrastructural analysis demonstrates a good preservation of plasma membrane till final apoptotic stages, the apoptotic cell appears unable to exclude PI when supravitally exposed to the dye. Fluorescence detection indeed shows a slow and progressive staining, indicating a possible membrane damage in cells entering the cell death program. When ultrastructural study is performed on the different cell fractions, this property appears typical of apoptotic cells and can be used as a criterion for their identification (Vitale *et al.*, 1993).

Finally, flow cytometry allows the correlation of apoptosis and cell cycle.

Electron microscopy

Transmission electron microscopy (TEM) of apoptotic cells can be performed by conventional embedding in epoxy resins or in acrylic ones (Falcieri *et al.*, 1994a). In the second case, it allows cytochemical approaches, such as osmium amine staining of nucleic acids (Derenzini and Farabegoli, 1990; Falcieri *et al.*, 1994a).

After appropriate chemical fixation immunocytochemical studies are also possible for the submicroscopic localization of apoptosis specific metabolites and gene products (De Jong *et al.*, 1994).

Legends for Figures 4 to 6 (on the facing page).

Figure 4. Agarose gel electrophoresis showing the DNA ladder of apoptotic cells. Lane **a** = Marker 123 bp ladder; Lane **b** = Control DNA; Lane **c** = DNA from apoptotic thymocytes; Lane **d** = Marker, ϕX 174 Hae III digested.

Figure 5. Agarose gel analysis of Molt-4 genomic DNA. One percent Agarose gel in 0.5 X TBE electrophoresis buffer was used. Lane **a** = Genomic DNA from untreated Molt-4 cells; Lane **b** = Genomic DNA from Molt-4 cells exposed to 20 nM staurosporine for 24 hours; Lane **c** = Lambda DNA/Eco RI + Hind III marker (the first band corresponds to 21226 bp).

Figure 6. PFGE: high molecular weight DNA was separated. Lane **a** = *S. cerevisiae* chromosomal DNA used as size marker; Lane **b** = Genomic DNA from untreated Molt-4 cells; Lane **c** = Genomic DNA from Molt-4 cells treated with 20 nM staurosporine for 24 hours; the first band corresponds to 2200 kb (arrow), the second one corresponds to 1300 kb (arrowhead).


A particular approach is represented by freeze-fracture (FF). This method, most commonly applied to the study of membrane and plasma associated subcellular structures (Falcieri *et al.*, 1990), appears to be quite amenable to apoptosis investigations. FF of nuclear membranes constantly shows a characteristic translocation of nuclear pores (Falcieri *et al.*, 1994b). This phenomenon, correlated to chromatin modification, appears typical of the apoptotic cell and can be clearly demonstrated by FF, which allows to clearly investigate nuclear membranes too. This nuclear envelope rearrangement can be also considered a marker of apoptotic cell. For this reason, the FF analysis of its nuclear components, when inner nucleus fracture occurs, is possible. Chromatin conformation studies can so be undertaken (Stuppia *et al.*, 1994).

Scanning electron microscopy (SEM) is also a powerful method to study programmed cell death, when surface alterations, probably correlated to cytoskeleton changes, appear (Liepins, 1989; Nagle *et al.*, 1990).

Results

MOLT-4 leukaemia cells, when treated with staurosporine, a protein kinase C blocking agent, show the apoptotic patterns, consisting in the progressive margination of chromatin towards nuclear poles (Fig. 3). Successively, uniformly electron dense micronuclei appear, characteristic of most apoptotic models.

Nevertheless, when DNA agarose gel electrophoresis was performed, this deep chromatin rearrangement
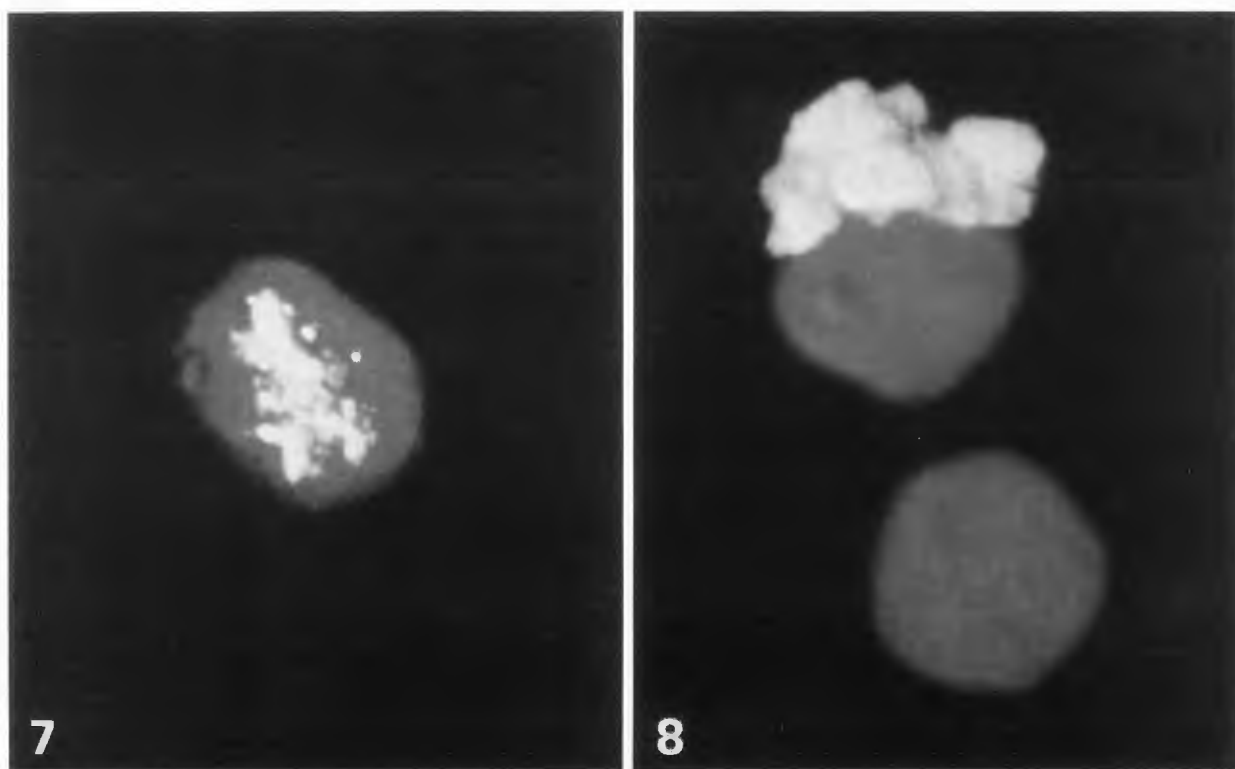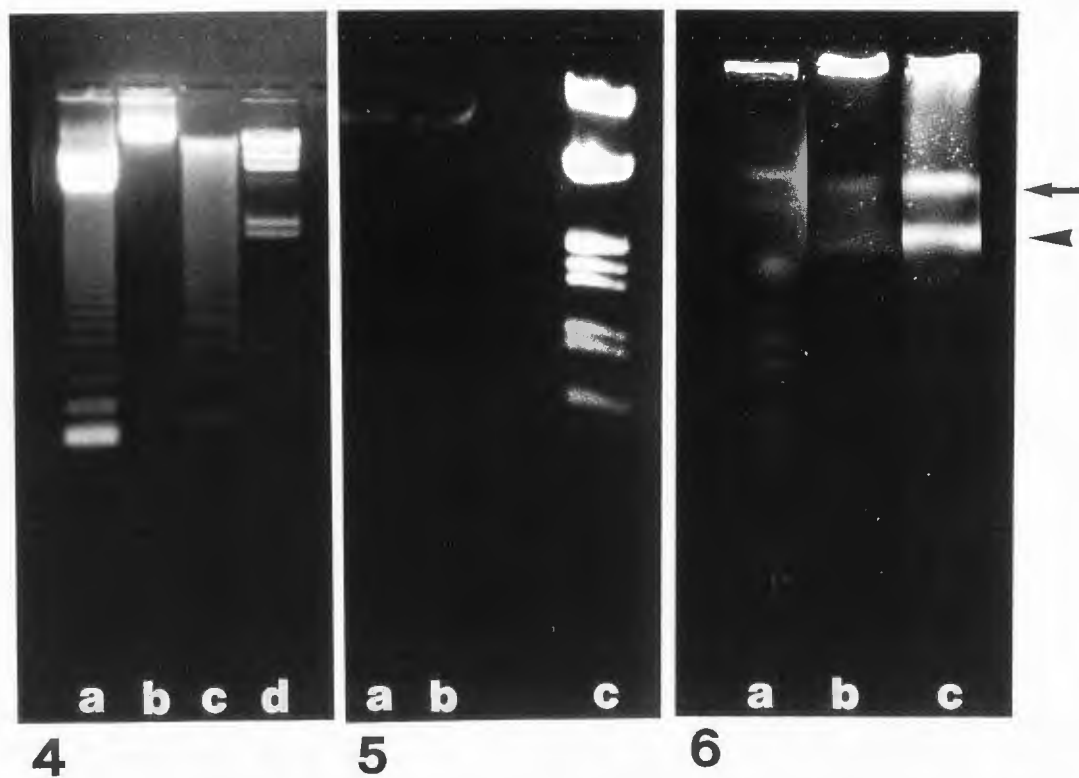
Figures 7 and 8. Methotrexate-treated HL 60 cells, "*in situ*" labelling of cleaved DNA. **Figure 7:** By TdT: a fluorescent central area appears, surrounded by PI stained DNA. **Figure 8.** By Pol I: a cap-shaped positive area appears at a nuclear pole; a non-apoptotic nucleus is present also.
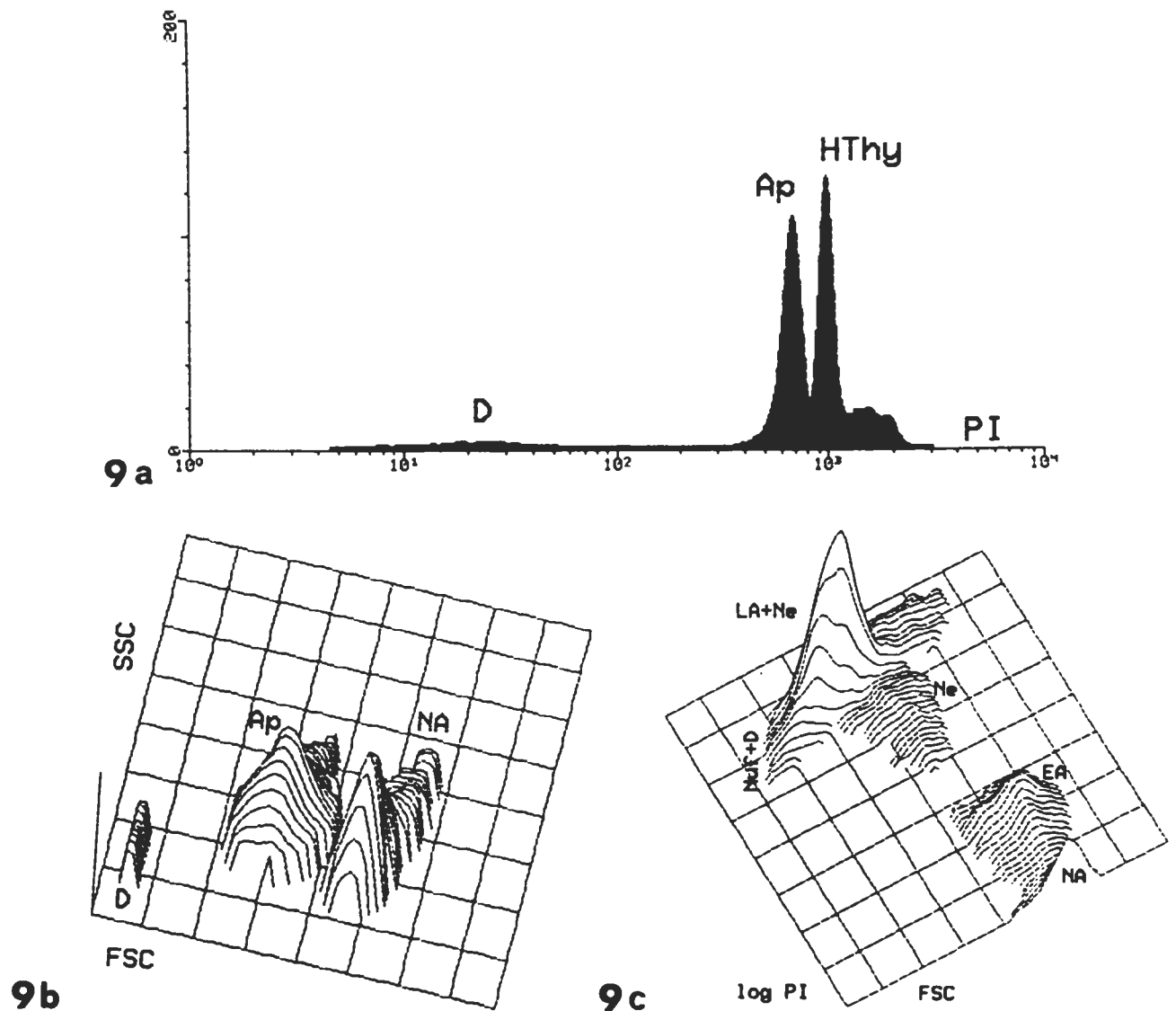
Figure 9. Flow cytometric patterns of apoptotic thymocytes. (a) A subdiploid peak is clearly identifiable after PI incubation of ethanol treated cells. Ap: apoptotic cells; HThy: healthy thymocytes; D: debris. (b) Apoptotic (Ap) and non-apoptotic (Na) cell populations, as well as debris (D) can be distinguished by means of the side scatter parameter. (c) Progressive PI uptake can be noted in fresh thymocytes undergoing apoptosis. NA: non-apoptotic cells; EA: early apoptosis; Ne: necrosis; LA + Ne: late apoptosis + necrosis; Nuf + D: nuclear fragments + debris.

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did not appear correlated to the DNA oligonucleosomic fragmentation (Falcieri *et al.*, 1993), such as the one commonly observed (Fig. 4).

The absence of DNA cleavage is demonstrated by the agarose gel electrophoresis, which appears negative (Fig. 5).

On the contrary, PFGE, performed to investigate different patterns of DNA fragmentation, revealed the presence of 2200 and 1300 kb fragments. The amount of cleaved DNA observable in the control is due to a number of apoptotic cells, which can be commonly iden-

tified in normal cells, potentially committed to apoptosis (Fig. 6).

In situ labelling of cleaved DNA, was analyzed by TdT and Pol I, in methotrexate-treated HL60 cells (Figs. 7 and 8). The behaviour of uncleaved DNA is shown by the PI stained normal nucleus visible in Figure 8. Differently, fluorescent areas correspond to the zones of fragmented DNA, which apparently reveals different patterns and localization with the two labelling methods. The TdT label (Fig. 7) presents indeed a fluorescent central area, surrounded by PI stained peripheral DNA.
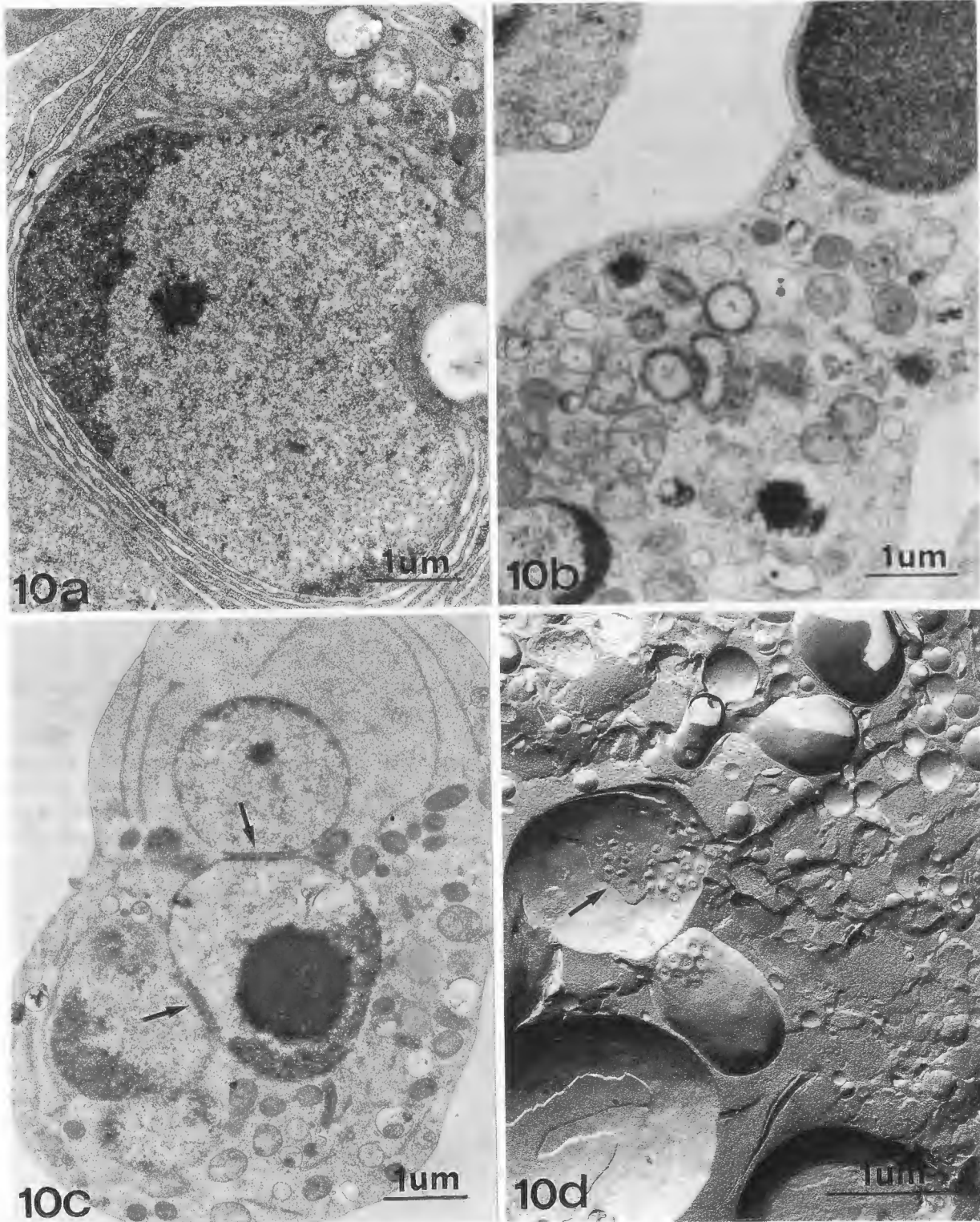
Figure 10. Transmission electron micrograph showing cell structures typical of apoptosis. A number of rough endoplasmic reticulum cisternae surround nuclear envelope (**a**). *"Annulate lamellae"* can be present (**b**). Nuclear pore clustering (arrows) is shown by conventional (**c**) and FF (**d**) TEM.

The Pol I label (Fig. 8) appears a fluorescent cap at a nuclear pole.

The different flow cytometric approaches are shown by Figure 9 in dexamethasone treated thymocytes undergoing programmed cell death. Figure 9a shows the subdiploid peak in PI incubated dexamethasone-treated thymocytes. When cells are treated with ethanol, plasma membrane undergoes a strong permeabilization. When low weight DNA is present, such as in apoptotic cells, a DNA loss occurs, accounting for the hypodiploid peak; thus, the presence of this peak can be considered an indication of the apoptotic cell population, and its size is related to their number. Figure 9b shows the scatter analysis with forward scatter, correlated to cell size, on x axis, and side scatter, a function of inner cell complexity, on y axis. Apoptotic and non-apoptotic thymocytes appear, in this case, as two clearly different populations.

Figure 9c describes the PI uptake in fresh thymocytes. When cells progressively undergo apoptosis, they are unable to exclude it and PI fluorescence (y axis) increases with time. Also, this approach can provide a tool for the identification of apoptosis, whose aspects can be further studied by TEM, easy and quick to perform in fresh cells.

Figure 10 shows different TEM features of HL60 apoptotic cells. Figure 10a shows the presence of numerous concentric lamellae of the rough endoplasmic reticulum surrounding the nucleus. In Figure 10b, stacks of *annulate lamellae* can be observed in the cytoplasm in late apoptotic stages. These two aspects can be correlated to nuclear envelope involvement in apoptotic nucleus rearrangement and can be found in apoptotic cells and, very rarely, in other metabolic conditions.

Nuclear pore translocation appears, by conventional TEM, in Figure 10c, and, by FF, in Figure 10d. It is present, to such an extent, only in apoptotic cells.

Discussion

The reproducible evidence of apoptotic features in presence of different and apparently contrasting metabolic events seems somehow intriguing. Moreover, the possibility to induce programmed cell death both interacting with membrane or nuclear receptors, as well as via different mechanisms, suggests the involvement of various metabolic pathways in this phenomenon.

Thus, the use of different technical approaches appears important to clarify the biochemical events underlying nuclear rearrangements.

Electrophoretical methods seem to support the fact that, even if DNA cleavage is not necessarily followed by the apoptotic changes (Fady *et al.*, 1994), it is anyhow present when the apoptotic picture appears. It oc-

curs in different ways in the various apoptotic models that requires the utilization of new investigation techniques (Brown *et al.*, 1993; Oberhammer *et al.*, 1993a,b; Tomei *et al.*, 1993).

Variable patterns of DNA cleavage can thus be revealed. Even when DNA ladder is not clearly shown by agarose gel electrophoresis, PFGE shows high molecular weight DNA fragments. From preliminary results, the DNA behaviour appears independent of the trigger and closely correlated to the cell type undergoing apoptosis (Cinti *et al.*, 1995).

How and if DNA fragment size influences cell changes is still unknown. Single strand and double strand breaks commonly appear within an apoptotic cell system.

DNA-polymerase NT technique seems more sensitive than TEM, revealing DNA breaks prior to the TEM related changes (Falcieri *et al.*, 1994a). TdT method, by contrast, exclusively labels the double strand breaks, and thus, gives different indications of apoptotic sources. It appears less sensitive than Pol I and frequently suggests a reorganization of cleavage points in larger and less numerous patches.

The DNA fragmentation mechanism is, thus, in some way, correlated to a spatial arrangement, but the real relationship to chromatin changes has still to be clarified (Gavrieli *et al.*, 1992; Fehsel *et al.*, 1993; Gorczyca *et al.*, 1993a,b; Wijsman *et al.*, 1993).

Flow cytometry is essentially the only method allowing the quantification of the apoptotic cell population, which can not be provided by the other techniques (Darzynkiewicz *et al.*, 1992). It represents a useful approach, preliminary to electron microscopy techniques, and crucial when cytochemical and immunocytochemical methods, requiring a certain amount of apoptotic cells, are employed. It allows the identification and the study of the apoptotic cell population by different approaches, based on different utilization of the cell sorter. The quantification of DNA loss consequent to fragmentation is possible, as well as information about apoptotic cell percentage (Zamai *et al.*, 1993). Data about the inner cell complexity strongly variable in the course of apoptosis, can also be obtained, and, even if in presence of a bad preservation due to ethanol fixation, TEM investigation can be performed (Zauli *et al.*, 1994). Flow cytometry technique also reveals characteristic apoptotic membrane patterns in the course of fluorochrome treatments (Vitale *et al.*, 1993).

By means of electron microscopy, progressive ultrastructural changes can be described in the apoptotic cell, as well as the appearance of aspects typical of it (Dabauvalle *et al.*, 1991; Gobbi *et al.*, 1994).

Nuclear pore clustering has been characterized by FF and can be considered an ultrastructural marker of

the apoptotic cell. It can be correlated to the deep rearrangement of apoptotic nuclear envelope closely correlated to a focal preservation of nuclear-cytoplasmic traffic (Falcieri et al., 1994b). It gives the possibility to reliably identify freeze-fractured apoptotic nuclei, where the chromatin rearrangement is virtually undetectable in FF. A criterion is thus stated to perform the morphometrical analysis of freeze-fractured chromatin, as previously reported in other experimental conditions (Marinelli et al., 1990). It also addresses new interest to nuclear envelope involvement in programmed cell death, as well as to nuclear pore structure and function.

Different technical approaches are therefore necessary to the understanding of apoptosis, whose intimate mechanisms are mostly still unknown.

Acknowledgements

References

Adolph F, Hameister H (1985) In situ nick translation of metaphase chromosomes with biotin-labelled dUTP. Histochemistry 69: 117-121.

Arends MJ, Wyllie AH (1991) Apoptosis: Mechanisms and roles in pathology. Int. Rev. Exp. Pathol. 32: 223-254.

Arends MJ, Morris RG, Wyllie AH (1990) Apoptosis: The role of the endonuclease. Am. J. Pathol. 136: 593-608.

Bøe R, Gjertsen BT, Vintermyr OK, Houge G, Lanotte M, Doskeland SO (1991) The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. Exp. Cell Res. 195: 237-246.

Brown GD, Sun XM, Cohen GM (1993) Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. J. Biol. Chem. 268: 3037-3039.

Cinti C, Santi S, Mariani AR, Columbaro M, Vitale M, Falcieri E (1995) Different patterns of DNA cleavage in MOLT-4 apoptotic cells. Eur. J. Histochem. 39: 48 (abstract).

Collins RJ, Harmon BV, Gobè GC, Kerr JFR (1992) Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. Int. J. Radiat. Biol. 61: 451-453.

Dabauvalle MF, Loos K, Merkert H, Scheer U (1991) Spontaneous assembly of pore complex-containing membranes ("annulate lamellae") in Xenopus egg extract in the absence of chromatin. J. Cell Biol. 112: 1073-1082.

Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hots MA, Lassota P, Traganos F (1992) Features of apoptotic cells measured by flow cytometry. Cytometry 13: 795-808.

De Jong D, Prins FA, Mason DY, Reed JC, Van Ommen GB, Kluin PM (1994) Subcellular localization of the bcl-2 protein in malignant and normal lymphoid cells. Cancer Res. 54: 256-260.

De La Torre J, Sumner AT, Gosalvez J, Stuppia L (1992) The distribution of genes on human chromosomes as studied by in situ nick translation. Genome 35: 890-893.

Derenzini M, Farabegoli F (1990) Selective staining of nucleic acids by osmium-amine complex in thin sections from Lowicryl-embedded samples. J. Histochem. Cytochem. 38: 1495-1501.

Dini L (1996) Phagocytosis of apoptotic cells. Scanning Microsc. 10: (this issue).

Fady C, Gardner A, Briskin K, Shmid I, Lichtenstein A (1994) A typical apoptotic cell death induced in L929 targets by exposure to tumor necrosis factor. Proc. Am. Assoc. Cancer Res. 35: 312 (abstract).

Falcieri E, Mariani AR, Mariani E, Gobbi P, Facchini A, Manzoli FA (1990) A morphological study of membrane lesions during natural killer-mediated lysis. J. Submicrosc. Cytol. Pathol. 22: 191-202.

Falcieri E, Martelli AM, Bareggi R, Cataldi A, Cocco L (1993) The protein kinase inhibitor staurosporine induces morphological changes typical of apoptosis in Molt-4 cells without concomitant DNA fragmentation. Biochem. Biophys. Res. Comm. 193: 19-25.

Falcieri E, Zamai L, Santi S, Cinti C, Gobbi P, Bosco D, Cataldi A, Betts C, Vitale M (1994a) The behaviour of nuclear domains in the course of apoptosis. Histochemistry 102: 221-231.

Falcieri E, Gobbi P, Zamai L, Faenza I, Vitale M (1994b) Nuclear pores in the apoptotic cell. Histochem. J. 26: 754-763.

Falcieri E, Gobbi P, Zamai L, Vitale M (1994c) Ultrastructural features of apoptosis. Scanning Microsc 8: 653-666.

Fehsel K, Kröncke KD, Kolb H, Kolb-Bachofen V (1994) In situ nick-translation detects focal apoptosis in thymuses of glucocorticoid and lipopolysaccharide treated mice. J. Histochem. Cytochem. 42: 613-619.

Gavrieli Y, Sherman Y, Ben-Sasson S (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. 119: 493-501.

Gobbi P, Zamai L, Rizzi E, Vitale M, Falcieri E (1994) Nuclear envelope behaviour in the course of

apoptosis. In: Proc. 13th Intl. Cong. Electron Microsc. (ICEM 13). Vol. 3A. Les Editions de Physique, Les Ulis, France. pp. 483-484.

Gorczyca W, Bigman K, Mittelman A, Ahmed T, Gong J, Melamed MR, Darzynkiewicz Z (1993a) Induction of DNA strand breaks associated with apoptosis during treatment of leukemias. Leukemia **7**: 659-670.

Gorczyca W, Gong J, Darzynkiewicz Z (1993b) Detection of DNA strand breaks in individual apoptotic cells by the *in situ* terminal deoxynucleotidyl transferase and nick translation assays. Cancer Res. **53**: 1945-1951.

Ito T, Hohjoh H, Sakaki Y (1993) Pulsed-field polyacrylamide gel electrophoresis: Basic phenomena and applications. Electrophoresis **14**: 278-282.

Kaufmann SH (1989) Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: A cautionary note. Cancer Res. **49**: 5870-5878.

Liepins A (1989) Morphological, physiological and biochemical parameters associated with cell injury: A review. Immunopharmacol. Immonotoxicol. **11**: 539-558.

Marinelli F, Falcieri E, Squarzoni S, Del Coco R, Zini N, Manzoli L, Maraldi NM (1990) Image analysis of the chromatin organization in the nuclear domains of freeze fractured hepatocytes and lymphocytes. Biol. Cell **70**: 107-119.

Nagle AW, Soloff BL, Moss AJ, Henle KJ (1990) Cultured chinese hamster cells undergo apoptosis after exposure to cold but nonfreezing temperatures. Cryobiology **27**: 439-451.

Oberhammer F, Fritsch G, Schmied M, Pavelka M, Printz D, Purchio D, Lasmann H, Schulte-Hermann (1993a) Condensation of the chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. J. Cell Sci. **104**: 317-326.

Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M (1993b) Apoptotic death in epithelial cells: Cleavage of DNA to 300 and/or 50 kb fragments prior to or in absence of internucleosomal fragmentation. EMBO J. **12**: 3679-3684.

Sambrook J, Fritsch EF, Maniatis T (1989) Pulsed-field gel electrophoresis from molecular cloning. In: Molecular Cloning: A Laboratory Manual. 2nd Edition. Book 1. Cold Spring Harbor Laboratory, New York. pp. 650-659.

Stuppia L, Gobbi P, Zamai L, Vitale M, Falcieri E (1994) Chromatin conformation in the apoptotic cell: A freeze-fracture morphometrical study. ICEM 13. *op. cit.* pp. 447-448.

Telford WG, King LE, Franker P (1992) Comparative evaluation of several DNA binding dyes in the de-
tection of apoptosis associated chromatin degradation by flow cytometry. Cytometry **13**: 137-143.

Tomei LD, Shapiro JP, Cope FO (1993) Apoptosis in $C_3H/10T\frac{1}{2}$ mouse embryonic cells: Evidence for internucleosomal DNA modification in the absence of double strand cleavage. Proc. Nat. Acad. Sci. **90**: 853-857.

Vitale M, Zamai L, Mazzotti G, Cataldi A, Falcieri E (1993) Differential kinetic of propidium iodide uptake in apoptotic and necrotic thymocytes. Histochemistry **100**: 223-229.

Walker PR, Pandey S, Sikorska M (1995) Degradation of chromatin in apoptotic cells. Death and Differentiation **2**: 97-104.

Wijsman JH, Jonker RR, Keijzen R, Van De Velde CJH, Cornelisse CJ, Van Dierendonck JH (1993) A new method to detect apoptosis in paraffin sections: *In situ* end-labeling of fragmented DNA. J. Histochem. Cytochem. **41**: 7-12.

Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: The significance of apoptosis. Int. Rev. Cytol. **68**: 251-306.

Zamai L, Falcieri E, Zauli G, Cataldi A, Vitale M (1993) The optimal detection of apoptosis by flow cytometry depends on cell morphology. Cytometry **14**: 891-897.

Zauli G, Vitale M, Re MC, Furlini G, Zamai L, Falcieri E, Gibellini D, Visani G, Davis BR, Capitani S, La Placa M (1994) *In vitro* exposure to human immunodeficiency virus type-1 (HIV-1) induces apoptotic cells death of the factor-dependent TF-1 hematopoietic cell line. Blood **83**: 167-175.

Discussion with Reviewers

Reviewer I: Could the authors speculate some more about the triggering induced by such varied stimuli? Is, for instance, protein synthesis necessary?

Authors: The paper is aimed at the presentation of different techniques to study apoptosis and the description of their potential. The biochemical events underlying the various apoptotic models are generally known and can be found in the current literature. We did not investigate the role of protein synthesis in the different cells undergoing experimental apoptosis.

Reviewer I: Do you think that other cell histotypes, such as epithelial or neuronal cells, could be studied by using the same approaches? Do they share similar pathways?

Authors: In principle, all the described techniques can be applied to epithelial, neuronal or other cell types. In the case of a tissue, cells can be separated by collagenase or trypsin and homogenized with a potter device or something similar. This procedure is necessary for

DNA electrophoresis and for flow cytometric analysis. NT techniques, as well as TEM, can be also performed on frozen or embedded sections. Similar morphological aspects can probably be demonstrated. On the contrary, different results could be revealed by gel electrophoresis and flow cytometry, as both techniques correlated to the way of DNA fragmentation, which can variously take place, as described.

V. Bumbaŝirević: What is your opinion about the competence of *in situ* nick-end labeling techniques for distinguishing between apoptosis and necrosis, because DNA cleavage also occurs during necrosis (although it is random and appears during the later stage)?

Authors: A necrotic cell, when prepared for NT, appears completely fluorescent, even if in an unspecific way. In apoptotic cells, chromatin marginations and micronuclei appear fluorescent and the general apoptotic morphology appears. It is the consistence of the different techniques, which proves the presence of apoptotic mechanisms.

V. Bumbaŝirević: The presence of *annulate lamellae* in the cytoplasm of apoptotic cells is an interesting finding. Was is regularly present in all apoptotic models that you have studied, and are they related to the nuclear envelope rearrangement during micronuclei formation in apoptotic cells?

Authors: *Annulate lamellae* have been regularly found in camptothecyn-treated HL60 cells and occasionally in the other apoptotic models. As previously described in some papers by Dabauvalle *et al.* (1991), they are related to the nuclear envelope rearrangement following chromatin margination and micronuclei formation (Falcieri *et al.*, 1994b).

T.M. Seed: The observation made principally by freeze-etching concerning nuclear pores during the apoptotic process is very interesting. Do you think this might be a structural manifestation of some sort of increased, but local informational exchange of essential molecules between apoptotic nuclear lobes and/or cytoplasm?

Authors: The translocation and clustering of nuclear pores is really an interesting and exclusive feature. In our paper (Falcieri *et al.*, 1994b), we discussed this point extensively. Our opinion is that it is correlated with the reorganization of metabolic exchange between different lobes of the apoptotic nucleus or between nucleus and cytoplasm. To prove this theory, a demonstration of pore functional state is necessary, and we are now investigating this. The regular contiguity of clustered pores to diffuse, possibly "active" chromatin areas

could also be correlated to a residual site of nuclear activity in the apoptotic cells. To test this hypothesis, experiments on chromatin are in progress.

L. Dini: How "typical" is the morphology of the dead cells during the process of apoptosis?

Authors: The morphology of dead cells is "typical" for this process because the nuclear behavior observed can be found exclusively in this model.

L. Dini: How can the morphological changes reflect the steps during the process of apoptosis and how can they be related to the apoptotic death?

Authors: Despite the variety of apoptotic inducers, the final stages of apoptosis appear closely comparable. At the moment, it is difficult to correlate an apoptotic trigger to a particular morphological event. A common mechanism is probably present, accounting for the ultrastructural pattern, even if stimuli are very different.

L. Dini: What is the influence of the condition of the cell (i.e., the stage in the cell cycle) in determining the pathway chosen towards apoptotic death?

Authors: A study on the relationship between apoptosis trigger and cell cycle is currently in progress.

L. Dini: Can you estimate the percentage of cells with DNA breaks revealed by DNA-polymerase NT technique that escape apoptosis?

Authors: The percentage of cells with DNA breaks that escape apoptosis can be estimated on control untreated NT processed cells and it is negligible. One has to consider that, in control specimens also, a very small percentage of apoptotic cells is constantly present.