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ULTRASTRUCTURAL FEATURES OF APOPTOSIS

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Abstract

Apoptosis is a gene-directed physiological and programmed process of cell deletion aimed at the regulation of tissue and organ development. It affects different cell types and is triggered by a variety of stimuli all inducing closely comparable structural changes. Despite the deeply different morphology and metabolism of the cell models and the various inducers and their initial effects, a convergence seems to take place in a common metabolic pathway that, in most cases, involves the activation of a Ca^{2+} dependent endonuclease. A growing body of data is now available on the molecular events that lead to DNA damage. DNA cleavage in nucleosomic or oligonucleosomic fragments is related to the appearance of unusual and very characteristic ultrastructural changes. The nucleus is especially affected, and shows chromatin rearrangements consisting of cup-shaped marginations, sharply separated from diffuse chromatin areas. Nuclear fragmentation subsequently appears, finally followed by the formation of numerous micronuclei. Cytoplasmic damage appears at a very late stage and the process takes place despite good preservation of plasma membrane and cytoplasm.

Key Words: Programmed cell death, apoptosis, necrosis, chromatin, ultrastructure.

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Introduction

Homeostatic control of cell number is the result of the dynamic balance between cell proliferation and cell death. However, only in recent years attention has been focused on the physiological occurrence of cell death and its role in the regulation of tissue proliferation. A kind of natural death, now called apoptosis, has then been identified. It is a wide-spread phenomenon, that plays a crucial role in numerous physiological events as well as pathological processes (Wyllie *et al.*, 1980; Arends and Wyllie, 1991; Schwartz and Osborne, 1993).

Morphological studies have revealed that cell death and removal by apoptosis is an active process during animal embryonic and foetal development.

Mechanisms such as blastocyst inner cell modelling, palatal fusion, interdigital web deletion, Müllerian duct regression, and motor neuron deletion in the absence of end-plate formation are regulated by the programmed death of particular cell populations (Ellis *et al.*, 1991; Garcia-Martinez *et al.*, 1993).

Apoptosis also plays a key role in metamorphosis and has been identified in amphibian tail loss and in caterpillar labial gland regression.

Normal tissue turnover is frequently modulated through apoptosis; hormone-dependent tissues represent an interesting and reproducible model to study the process, cell death being caused by the decrease of the circulating trophic hormone concentration. Thymus atrophy is considered the most common example of apoptosis and fresh or cultured thymocytes have long been utilized for experimental studies on the phenomenon (Moss *et al.*, 1985; Brune *et al.*, 1991; Cohen *et al.*, 1993). In addition, it has been observed in cyclic modifications of the endometrium, in breast proliferation and deletion, in ovary corpus luteum regression and in adrenal cortex and prostate atrophy (Walker *et al.*, 1989).

Also the growth factor-dependent survival of many cell types seems to be strongly modulated by apoptosis. IL-2 withdrawal from thymocytes, hemopoietic precursor culture in absence of IL-3, serum deprivation of fibroblast and other cell cultures lead to cell damage and

death with typical apoptotic features (Zamai *et al.*, 1993).

The turnover of normal adult tissues has frequently been correlated to programmed cell death. In the current view of hematopoiesis, pluripotent progenitor cells proliferate and differentiate into committed progenitors, which are restricted in their subsequent development to a single cell lineage. Hematopoietic growth factors play a major role in preventing apoptosis, allowing progenitor cells to survive and express their differentiation program.

The normal fate of progenitor cells deprived of specific growth factors is death and both molecular events and morphological patterns suggest apoptotic mechanisms (Koury, 1992).

Programmed cell death appears also to play an important role in immune cytotoxicity. Cytotoxic T, Natural Killer and K cells are able to commit their target to programmed death (Goldstein *et al.*, 1991; Cohen, 1991).

Apoptosis also seems to take place in the course of cell aging, as demonstrated by the appearance of typical patterns in senescent neutrophils in the course of inflammation (Savill *et al.*, 1989).

Cell deletion in cancer is also frequently related to programmed cell death. Characteristic nuclear and cytoplasmic changes have been described in basal and squamous cell skin carcinomas and in the regression of endocrine-dependent tumor. This behavior represents the basis of tumor cell death after treatment with tumor necrosis factors (Bellomo *et al.*, 1992) or cancer chemotherapeutic agents. Etoposide, camptothecin, cisplatin, 5-fluorouracil, methotrexate, teniposide, vincristine and many other anticancer agents have been reported to induce apoptosis in cultured tumor cells (Kaufmann, 1989; Sorenson *et al.*, 1990; Williams, 1991; Sen and D'Incalci, 1992).

The final stage of toxin-induced cell damage is frequently characterized by apoptotic features.

X-ray irradiation, at determined intensities and times, has also been described to induce apoptosis in cortical thymocytes, in intestinal mucosal cryptae, in Sertoli and cerebellar cells (Allan *et al.*, 1988; Harmon and Allan, 1988; Geng and Potten, 1990; Ijiri and Potten, 1990; Stephens *et al.*, 1991; Kruman *et al.*, 1991) as well as in other experimental models (Di Pietro *et al.*, 1994).

Hyperthermia seems to cause apoptotic changes, as recently described in a mastocytoma cell line (Collins *et al.*, 1992). Exposure to non-freezing temperatures can also induce apoptotic features (Nagle *et al.*, 1990).

Microorganisms have also been suggested to be the causal agent of programmed cell death. *Shigella flexneri* causes apoptotic changes in phagocytizing macrophages (Zychlinsky *et al.*, 1992). Also some viruses appear involved in apoptotic target cell phenomena (McCabe and Orrenius, 1992; Zauli *et al.*, 1994).

Apoptosis Versus Necrosis

When cells are subjected to a markedly injurious environment, a type of accidental cell death often occurs which is called necrosis. It can be caused by a wide variety of harmful conditions and toxic substances, including hyperthermia, hypoxia, ischemia, complement attack, metabolic poisons and direct cell trauma (Wyllie *et al.*, 1980; Ledda-Columbano *et al.*, 1991; Schwartzman and Cidlowski, 1993).

A cell undergoing necrosis typically exhibits distinctive morphological and biochemical characteristics (Fawthorp *et al.*, 1991). The earliest modifications consist of progressive hydratation of cytoplasm and organelles, with initially minimal morphological changes in the nucleus represented by focal chromatin margination. Plasma membrane and organelle disruption successively appear, allowing the lysosomal content to leak out into the cytoplasm. Finally, nuclear dissolution takes place and cell undergoes a profound disintegration (Fig. 1). The morphological changes are due to the loss of control of selective permeability of the plasma membrane (Falcieri *et al.*, 1990). These alterations are due to the early disappearance of membrane ion-pumping activities, consequent to both membrane damage (Falcieri *et al.*, 1992) and cell energy depletion (Trump and Berezesky, 1992). Cation movement across the membrane takes place, determining the water influx which causes the tremendous cell swelling. The ion-dependent activation and the release of numerous enzymes causes the disintegration of the cell in the final stage of the necrosis (Zychlinsky *et al.*, 1991). In tissues, it typically affects groups of contiguous cells and an inflammatory reaction usually develops in the adjacent cells in response to the released cell debris.

Apoptosis is a morphologically distinct cell death, that spontaneously occurs in many different tissues under various conditions. It occurs in distinctly separated cells and progresses very rapidly, never causing exudative inflammation in tissues. No cell hydratation takes place, but nuclear and cytoplasmic condensation can appear, followed by the formation of numerous membrane-bound cell fragments termed "apoptotic bodies". Differently from necrosis the nuclear organization is completely lost. Profound chromatin rearrangements take place, followed by the formation of a variable number of compact, electron dense micronuclei. Surprisingly, despite the extensive nuclear changes, both cytoplasm and organellar components remain intact for some time, unless the cell undergoes secondary necrosis. Only in the final

apoptotic stage, the whole cell is strongly involved and undergoes a "secondary" necrosis (Fig. 2). Apoptosis can be observed in many different tissues, both healthy and neoplastic, adult and embryonic, but normally does not coexist with necrotic death as it represents a completely different phenomenon.

Molecular Events of Apoptosis

Apoptosis is a gene-directed process. The model which best elucidated the genetic involvement in apoptosis is *Caenorhabditis elegans* (Williams and Smith, 1993). In this nematode, 1090 cells are formed from the fertilized zygote and a fixed number of these undergo death at different, but precisely defined, times (Ellis *et al.*, 1991). Two genes have been identified of which the activity is required to induce apoptosis. They have been named *ced-3* and *ced-4* (cell death genes 3 and 4). Mutations of these genes determine the survival of almost all cells that normally die (Williams and Smith, 1993). The proteins encoded by these two genes are now partially known and Ca^{2+} activation as well as protein phosphorylation seems to play a role in apoptotic molecular events (Schwartzman and Cidlowski, 1993).

The *ced-3* and *ced-4* genes appear to be negatively regulated by the *ced-9* gene (Ellis *et al.*, 1991), which protects cells that must survive from programmed cell death.

The *bcl-2* (B- cell lymphoma-2) human gene, has been subsequently demonstrated to prevent apoptosis when transfected into *C. elegans* (Alnemri *et al.*, 1992; Bissonnette *et al.*, 1992; Vaux *et al.*, 1992). Overexpression of *bcl-2* delays the onset of cell death induced by the deprivation of growth factors in several hemopoietic cell lines (Vaux, 1993). The *bcl-2* protein is localized to the intracellular sites of generation of oxygen free radicals, such as the inner mitochondrial membrane, the endoplasmic reticulum, and the nuclear membranes (Krajewski *et al.*, 1993).

bcl-2 gene has been suggested to protect cells from apoptosis by altering mitochondrial function (Jacobson *et al.*, 1993), and regulating antioxidant pathways at the sites of free radicals generations (Hockenbery, 1993). Recently, *bcl-2* has been shown to be involved in the prevention of apoptosis in T cells, thymocytes, germinal center B-cells, Burkitt lymphoma cells, and in pre-B leukemia lines, thus playing a key role in regulating mechanisms of the immune system (Sen and D'Incalci, 1992).

A recently identified 21 kD protein, Bax, has been shown to heterodimerize with *Bcl-2*, inhibiting its anti-apoptotic activity (Oltvai *et al.*, 1993). Several proto-oncogenes, normally involved in cell cycle regulation, are also involved in the induction of apoptosis. Virtually all apoptotic inducers that are active on S phase cells, require *c-myc* activation (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992; Shi *et al.*, 1992; Amati *et al.*, 1993). *c-fos* and *c-jun* expression is also often increased during apoptosis. It appears as if a cell committed to cell cycle will have to decide either to go on with DNA synthesis or to die depending on the integrated complex of external and internal signals (soluble factors, cell to cell contact, other gene expression) it receives. *c-myc* and *c-K-ras* expression are, on the contrary, inhibited in glucocorticoid-induced thymocyte apoptosis (Schwartzman and Cidlowski, 1993), which, however, takes place in the G_0 phase of the cell cycle. Premature p34 activation seems also required for apoptosis induction (Shi *et al.*, 1994). Specific gene activation represents an early event during apoptosis that, however, appears dependent on signal transduction to the nucleus, generated by numerous and completely different agents.

These "apoptotic factors" act in two different ways, depending on their biochemistry and on target cell behavior (Fesus *et al.*, 1991; Schwartzman and Cidlowski, 1993). They can interact with surface receptors, initiating apoptosis via signals induced by second messenger molecules (Cory, 1994). Other factors, such as glucocorticoids-which trigger thymocyte apoptosis, and represent one of the best known apoptotic models-interact with nuclear receptors. These receptors activate the transcription of genes linked to the induction of the apoptotic process. Apoptosis also appears after loss of trophic signals, that normally suppress the expression of the cell death program. Cytokines, growth factors, hormones and other stimuli behave as "survival factors" through either cell surface or nuclear receptors (Fesus *et al.*, 1991; Williams, 1991; Schwartzman and Cidlowski, 1993).

The final molecular event is, in most cases, the activation of a Ca^{2+} dependent endonuclease (Arends *et al.*, 1990; Giannakis *et al.*, 1991; Trump and Berezesky, 1992; Tomei *et al.*, 1993). This enzyme, generally inactive, becomes active in a still partially unknown way when programmed cell death is triggered (Peitsch *et al.*, 1993). The final effect is, however, DNA cleavage in nucleosomic or oligonucleosomic fragments (180 base pairs or multiples) that can be detected by agarose gel electrophoresis and is generally considered the hallmark of apoptotic cell death (Fig. 3). This phenomenon is common to most apoptotic models, even if recently the absence of DNA cleavage, or possibly different breaking mechanisms in the presence of the usual morphological changes (Collins *et al.*, 1992; Oberhammer *et al.*, 1993; Falcieri *et al.*, 1993), as well as a DNA ladder with no apoptotic ultrastructural features (Fady *et al.*, 1994), occasionally have been reported. Other methods for quantification and measurement of apoptosis are based

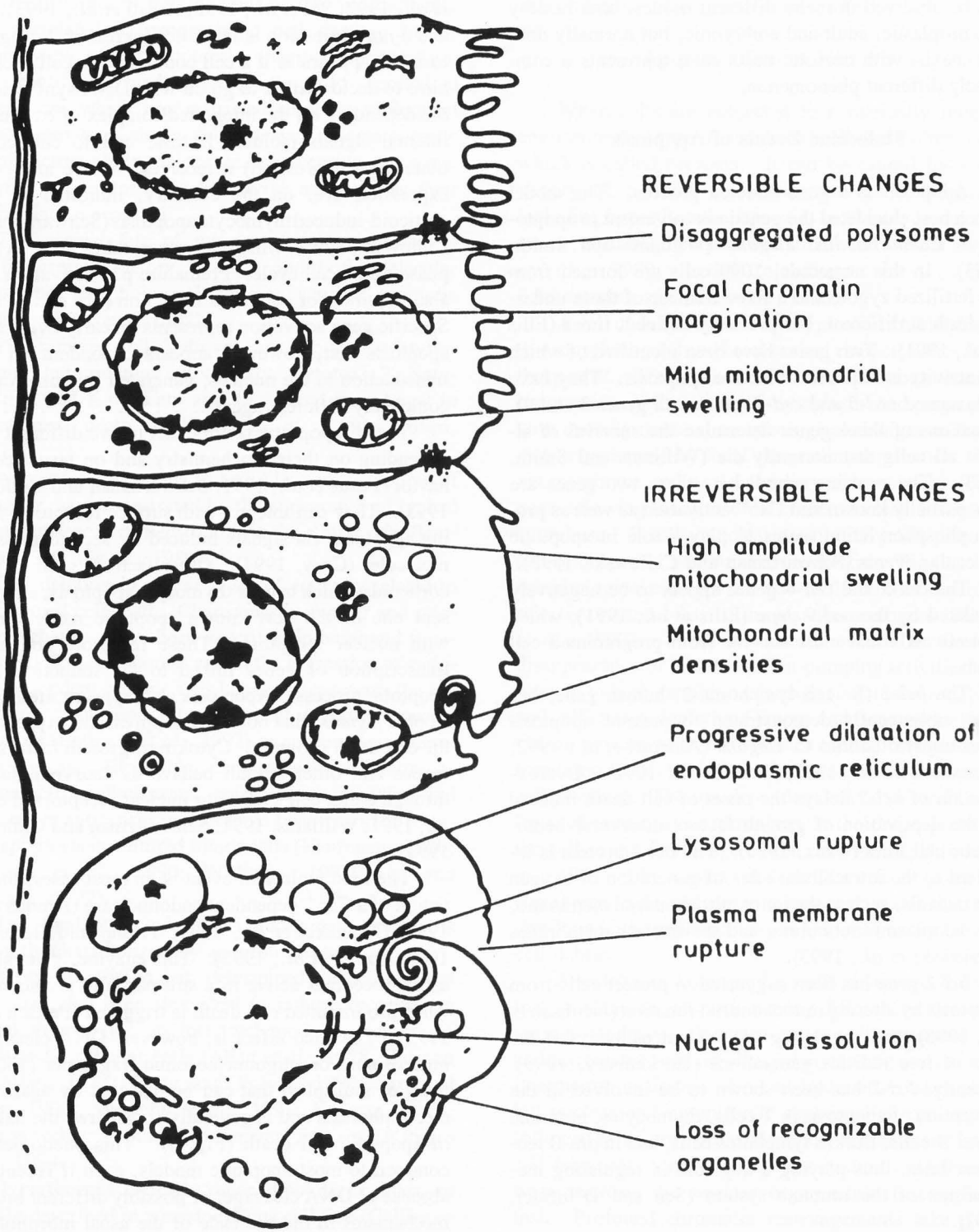
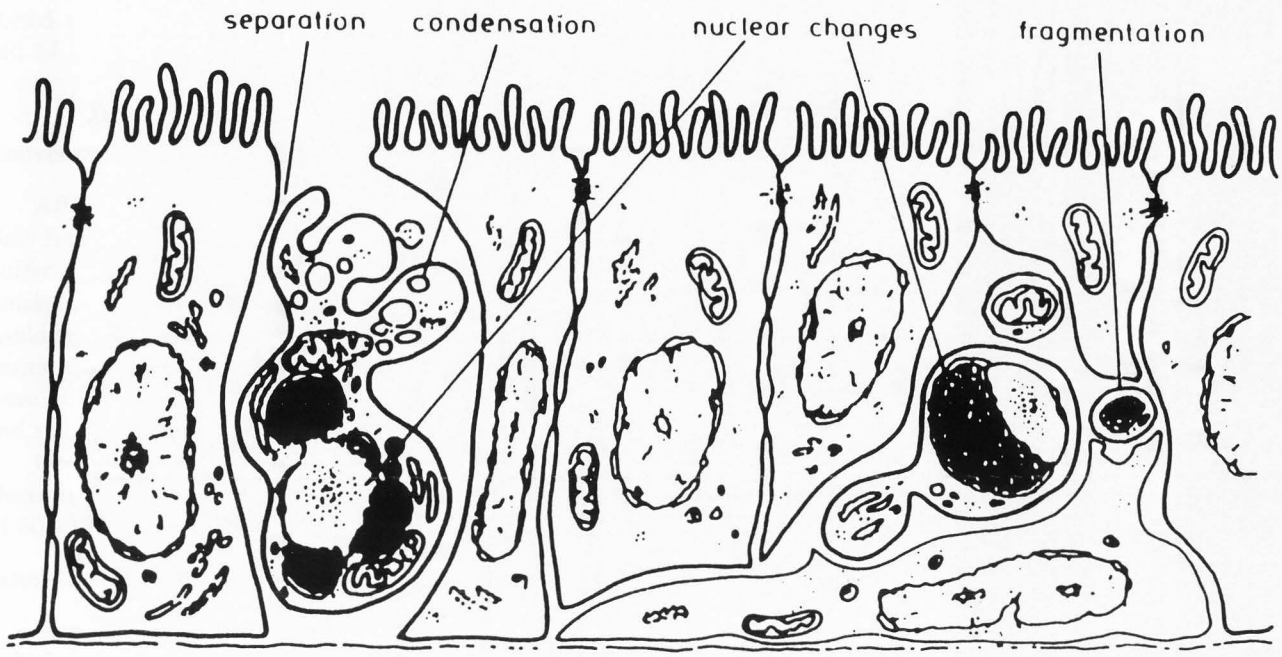


Figure 1. Morphological characteristics of necrosis (from Bowen and Bowen, 1990).



FATE OF APOPTOTIC CELLS

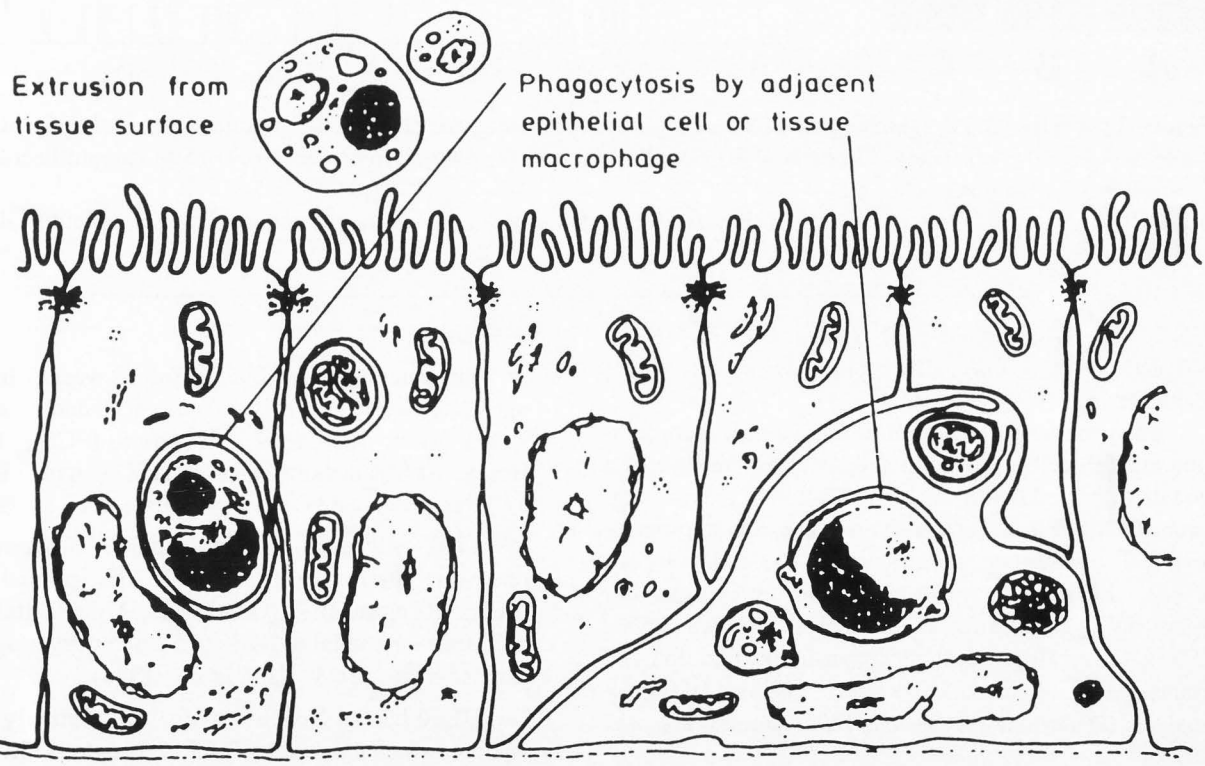


Figure 2. Morphological characteristics of apoptosis (from Bowen and Bowen, 1990).

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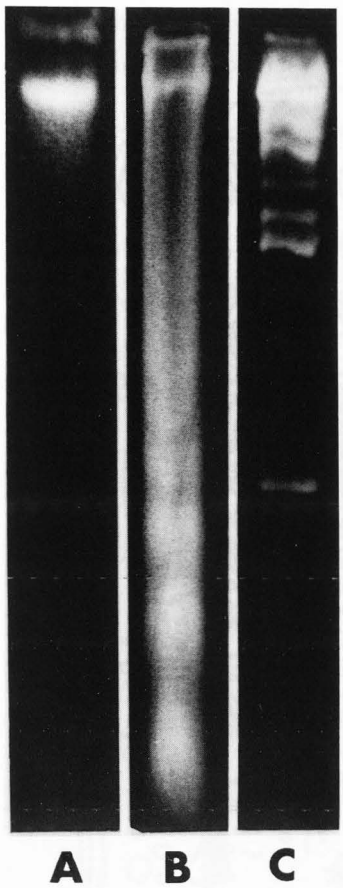
on DNA fragmentation and loss. DNA specific fluorochromes (propidium iodide, Dapi; acridine orange, Hoechst and others) allow the identification by flow cytometry analysis of cells with decreased amounts of



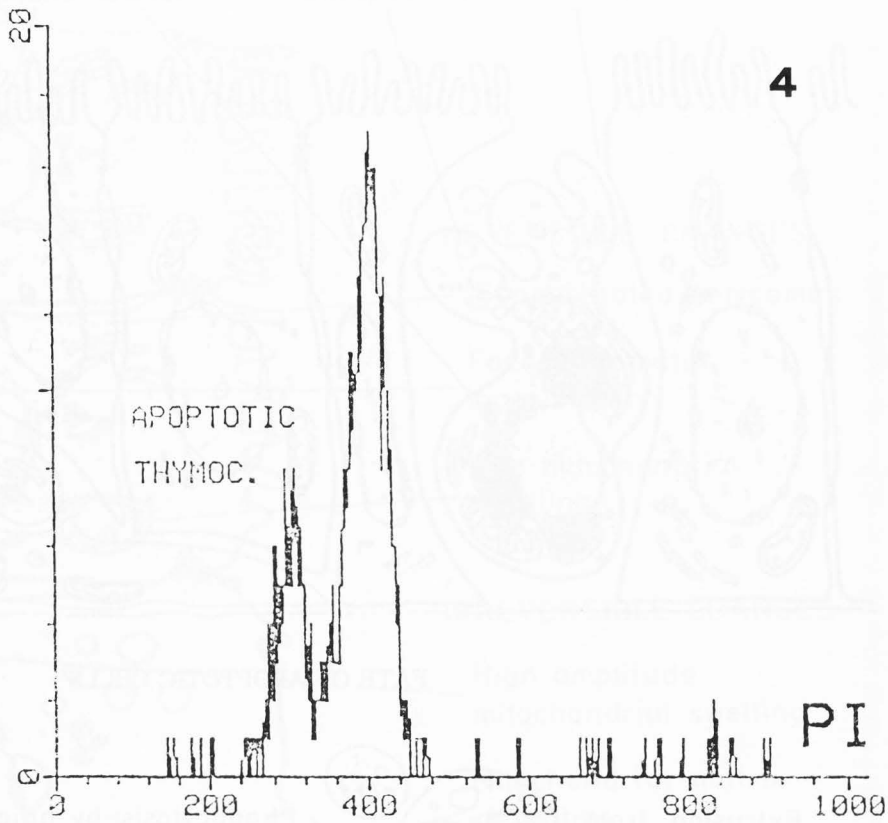


Figure 3 (at left). 0.8% agarose gel DNA electrophoresis, showing the characteristic ladder pattern of apoptotic cells. **Lane A** = HL60 control cells. **Lane B** = HL60 cells, cultured for 6 hours in presence of 0.15 M camptothecin. **Lane C** = molecular weight marker III.

Figure 4 (at right). Flow cytometry of thymocytes treated with 10^{-7} M dexamethasone: the hypodiploid peak corresponds to the apoptotic cell population.

DNA as a hypodiploid peak (Telford *et al.*, 1992; Nicoletti *et al.*, 1991; Darzynkiewicz *et al.*, 1992; Zamai *et al.*, 1993) (Fig. 4).

The peculiar pattern of propidium iodide incorporation in fresh cells induced to apoptosis also represents a quantitative method for the identification of the phenomenon, the late phases of which are correlated to progressive cell membrane permeabilization (Vitale *et al.*, 1993). Finally, flow cytometry can help to quantify apoptosis by scattering signal analysis (Zamai *et al.*, 1993), or by fluorescent nick translation (Adolph and Hameister, 1985; De la Torre *et al.*, 1992) or TdT assay analysis (Darzynkiewicz *et al.*, 1992; Gorczyca *et al.*, 1993).

Materials and Methods

Cells

In our experiments the following apoptotic models were used:

Thymocytes were obtained from 2 weeks old Balb/c mice (Charles River, Milano) and incubated 24 hours in RPMI 1640, 10% fetal calf serum (FCS), 10 mM Hepes, 10^{-7} M dexamethasone (DEX) (Sigma, U.K.) at 37°C as previously described (Vitale *et al.*, 1993).

TF-1 cells, a human hematopoietic progenitor cell line, were cultured in RPMI 1640, 10% fetal calf serum (FCS), 10 ng/ml IL-3 (Omnia Res, Milano, Italy), and thereafter kept without IL-3 for 72 h to trigger apoptosis (Zamai *et al.*, 1993; Zauli *et al.*, 1994).

HL60 human leukemia cells were routinely grown. Apoptosis was induced by 0.15 M camptothecin (CAM) (Sigma, U.K.) for 6 hours at 37°C 5% CO_2 (Kaufmann, 1989; Darzynkiewicz *et al.*, 1992).

Human lymphocytic leukemia MOLT-4 cells (ATCC, Rockville, MD, USA) were cultured in RPMI 1640, induced to apoptosis by addition of staurosporine

(Boehringer, Mannheim, Germany), as previously reported (Falcieri *et al.*, 1993), and observed between 6 and 24 hours of treatment.

Untreated cells were utilized as control specimens.

Conventional electron microscopy

After incubation, cells were sedimented and immediately fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer. They were then postfixed with 1% OsO_4 in veronal buffer, dehydrated in ethanol and embedded in Araldite. Toluidine blue-stained semi-thin sections permitted identification of apoptotic cells. Thin sections were collected on Formvar-carbon coated nickel grids and stained with uranyl acetate and lead citrate.

Observations were carried out with a Philips CM10 electron microscope operated at an accelerating voltage of 80 kV.

Scanning electron microscopy

The analysis was carried out by cell deposition on poly-L-lysine coated cover slips after fixation with 2.5% glutaraldehyde in 0.1 M phosphate buffer. Samples were postfixed with OsO_4, dehydrated in ethanol, critical point dried and gold sputtered.

A Philips 515 scanning electron microscope was utilized for the observations.

Ultrastructural Features

Despite the variety of experimental models, the behavior of the different cells, during the apoptotic phenomenon, seems to be morphologically comparable. Ultrastructural studies clearly substantiate a common pattern of involvement of different subcellular components during apoptosis. The process has been long investigated by electron microscopy "*in vivo*" (Wyllie *et al.*, 1980; Arends and Wyllie, 1991; Kawabori *et al.*, 1991) as well as "*in vitro*" (Arends *et al.*, 1990; Sorenson *et al.*, 1990; Ledda-Columbano *et al.*, 1991; Lazebnik *et al.*, 1993). The induction of apoptosis in differently treated cell lines in which the morphological approach can be supported by molecular biology assays allowed to clarify many details of the process.

Scanning electron microscopy suggested an involvement of cell membrane in the phenomenon, consequent to the cytoskeleton rearrangement due to the deep nuclear changes. Blebs and rufflings indeed appear on the plasma membrane (Allan *et al.*, 1988; Harmon and Allan, 1988).

In any case, the first morphological alterations are shown by the nucleus and, particularly, the chromatin (Falcieri *et al.*, 1994a). This progressively clusters and wide areas of condensed chromatin appear marginated, at first close to the nuclear envelope (Fig. 5a), and then in well defined cap-shaped formations at the nuclear poles (Fig. 5b). These structures appears uniformly electron dense and sharply separated from the diffuse chromatin zones (Falcieri *et al.*, 1993; Zamai *et al.*, 1993) (Fig. 5c). At this stage, despite the evident changes in chromatin, the nucleus mostly maintains its topography and correlations with cytoplasmic districts. The nucleolus as well as the nuclear envelope and the other nuclear domains are generally well recognizable. In a subsequent phase nuclear protrusions appear (Fig. 5d), with a profound rearrangement of the nuclear membranes. Finally, micronuclei are formed, in most cases surrounded by a double membrane externally outlined by regularly distributed ribosomes (Fig. 5e). The micronuclei can be numerous and contain a compact, homogeneously electron dense chromatin (Vitale *et al.*, 1993). Occasionally they are initially formed in the perinuclear space (Fig. 5f), but more frequently, they appear scattered throughout the cytoplasm (Fig. 6a). When the process takes place in polymorphic cells, with irregular nuclei, such as HL60 leukemic cells, that frequently release cleaved DNA into the cytoplasm, micronuclei do not appear homogeneously electron dense (Fig. 6b).

The cytochemical approach, such as osmium ammine selective staining, or DNase-colloidal gold complex technique allow the differential localization of nucleic acid during the phenomenon and suggest the almost exclusive presence of DNA in cap-shaped areas and in micronuclei (Falcieri *et al.*, 1994a, b).

Surprisingly, also in these so called "apoptotic bodies", which are quite different from the original cells, both the plasmalemma and the organelles appear well preserved under a long time (Figs. 6c and 6d). During the late apoptotic stages "secondary necrosis" patterns appear, characterized by hydration of the cytoplasm, membrane disruption and cell debris (Fig. 6e). The final fate of the apoptotic cell is a complete disintegration or the phagocytosis by contiguous or circulating cells (Fig. 6f). The extrusion of micronuclei into the extracellular space can be frequently observed in thymocytes (Fig. 7a) and hemopoietic cells (Fig. 7b).

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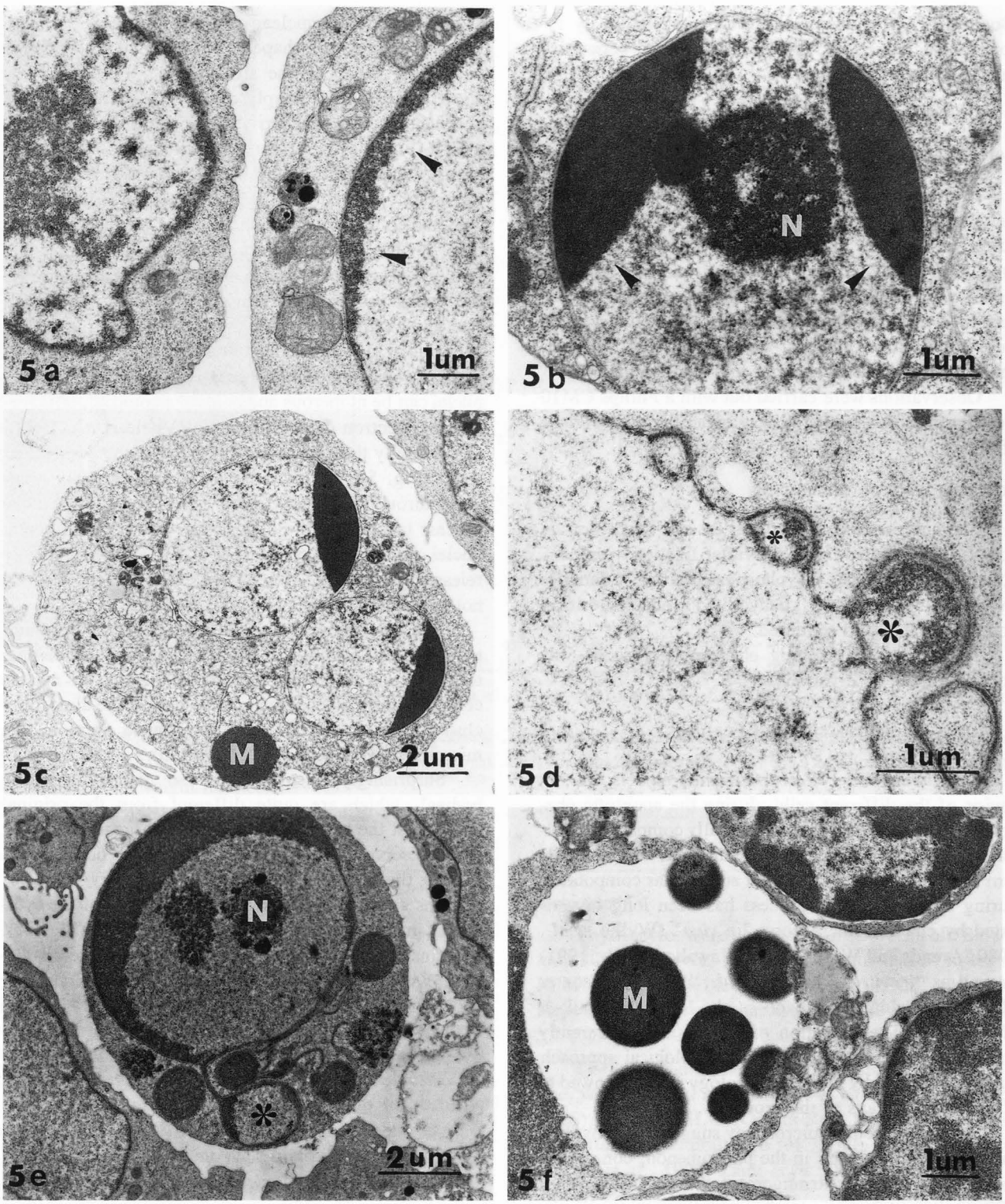

Figure 5. Ultrastructural aspects of apoptotic cells. The early chromatin clusters (**a**) are followed by more dense chromatin marginations (**b**), sharply separated from diffuse areas. Uniformly electron dense micronuclei (**M**) also appear (**c**). They can protrude from the nuclear periphery (**d**, and **e**), often involving large portions of nuclear membrane. Differently (**f**), they (**M**) can be assembled in the perinuclear cisternae. Nucleolar components can be frequently recognized in the diffuse chromatin areas (**N**). a and d = Molt 4 cells; b, c and e = TF1 cells; and f = thymocytes. **Bars** = 1 μm, except in 5c and 5e, where bar = 2 μm. (From Falcieri *et al.*, 1993; Zamai *et al.*, 1993).

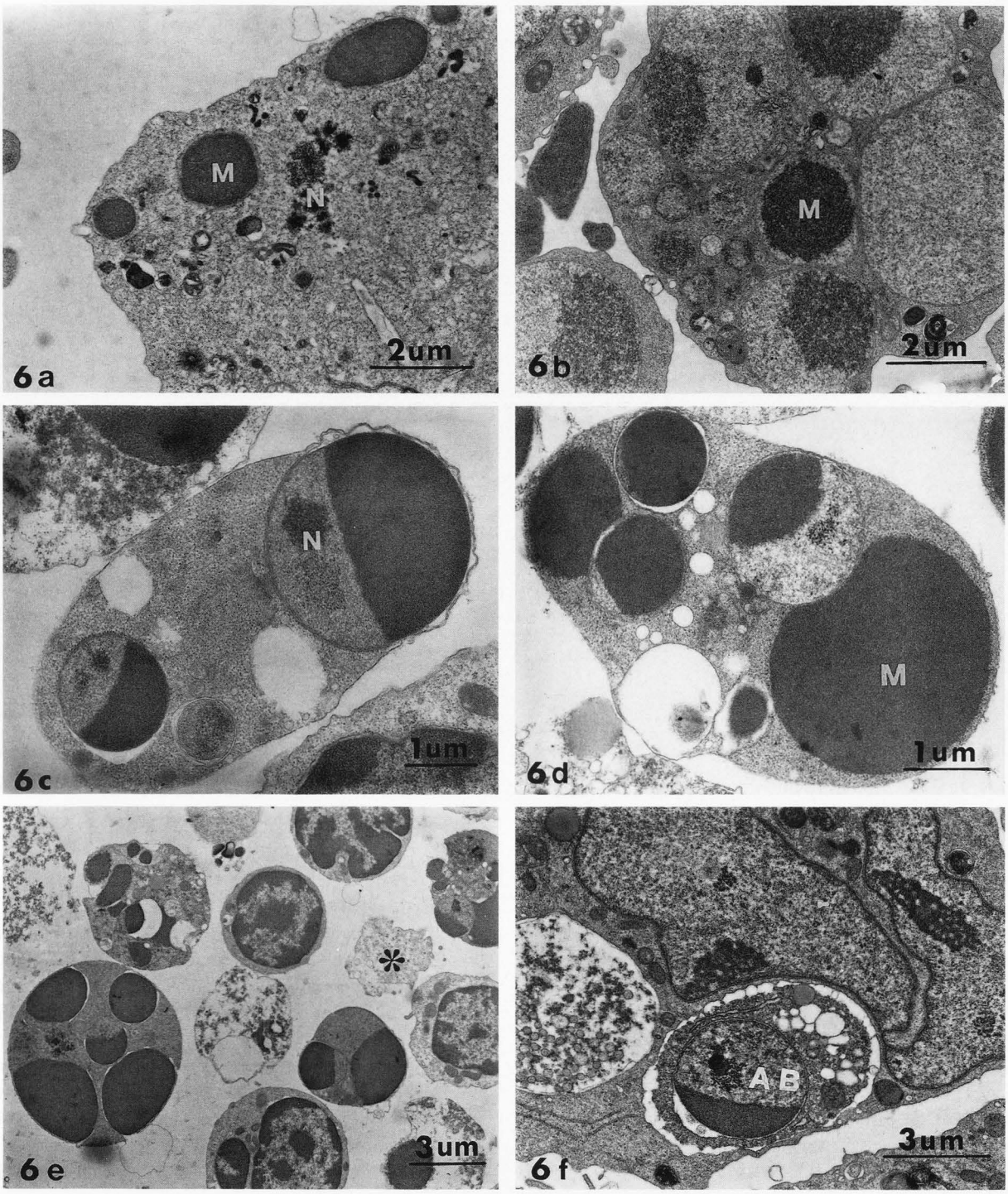

Figure 6. Further advanced apoptotical features. Numerous micronuclei (**M**) appear scattered throughout the cytoplasm (**a, b, c, d,** and **e**). They contain homogeneously compact chromatin (**a, d, e**), irregularly clustered chromatin (**b**), or the typical cap-shaped electron dense structures (**c**). Nucleolar components (**N**) appear in the diffuse chromatin areas (**c**) or occasionally scattered in the cytoplasm (**a**). Despite these strong irreversible nuclear changes, the plasma membrane and the organellar component appear long well preserved. Necrotic aspects (**e**) or phagocytized apoptotic bodies (**AB**) appear in the very late stages of the process (**f**). a = Molt 4 cells; b = HL60 cells; c, d and e = thymocytes; and f = TF1 cells. Bars = 2 μm (in a and b); 1 μm (in c and d); and 3 μm (in e and f). (From Falcieri *et al.*, 1993; Zamai *et al.*, 1993; Vitale *et al.*, 1993).

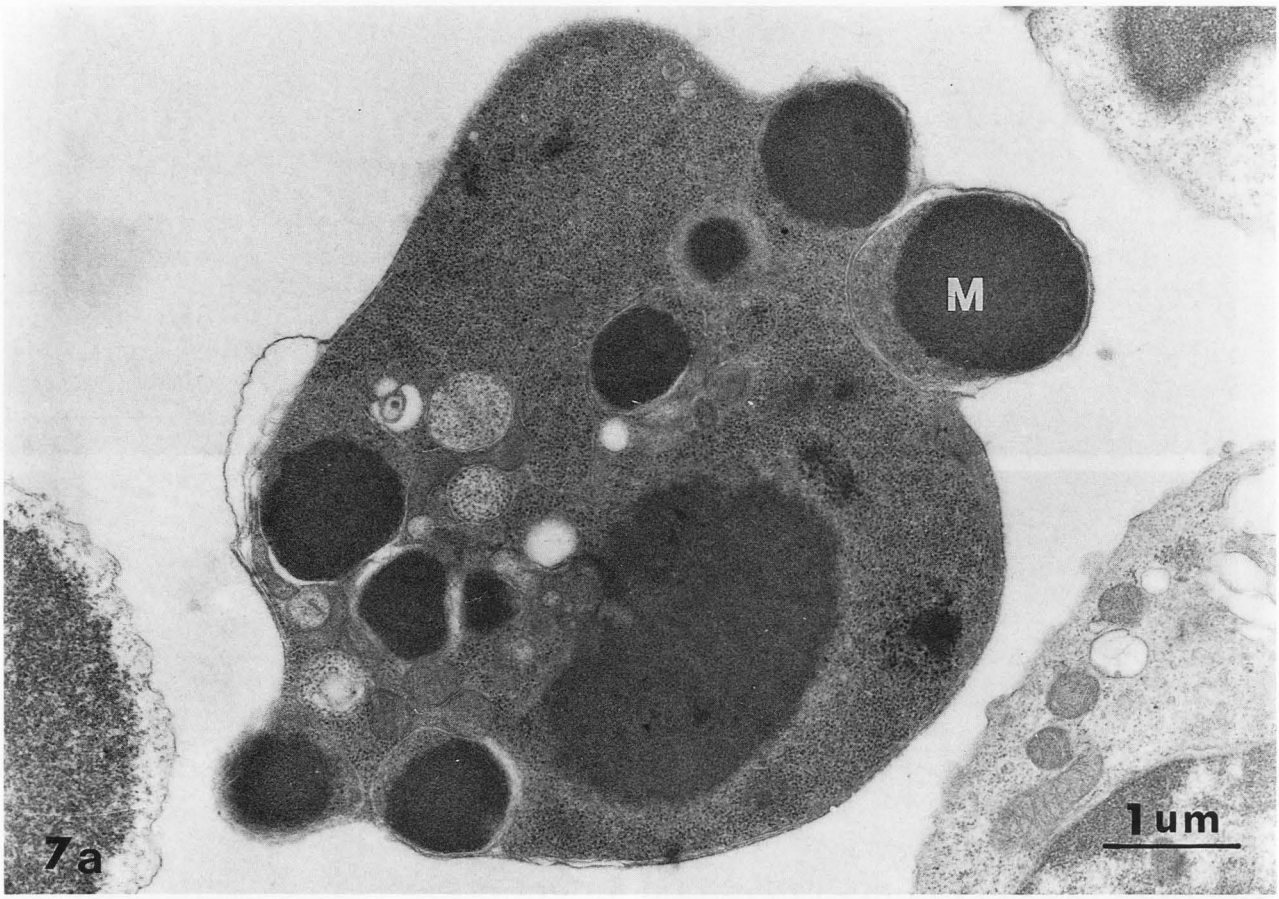

Figure 7a. Transmission electron micrographs of micronuclear extrusion from late apoptotic thymocyte cells. (from Vitale *et al.*, 1993). Bar = 1 μm.

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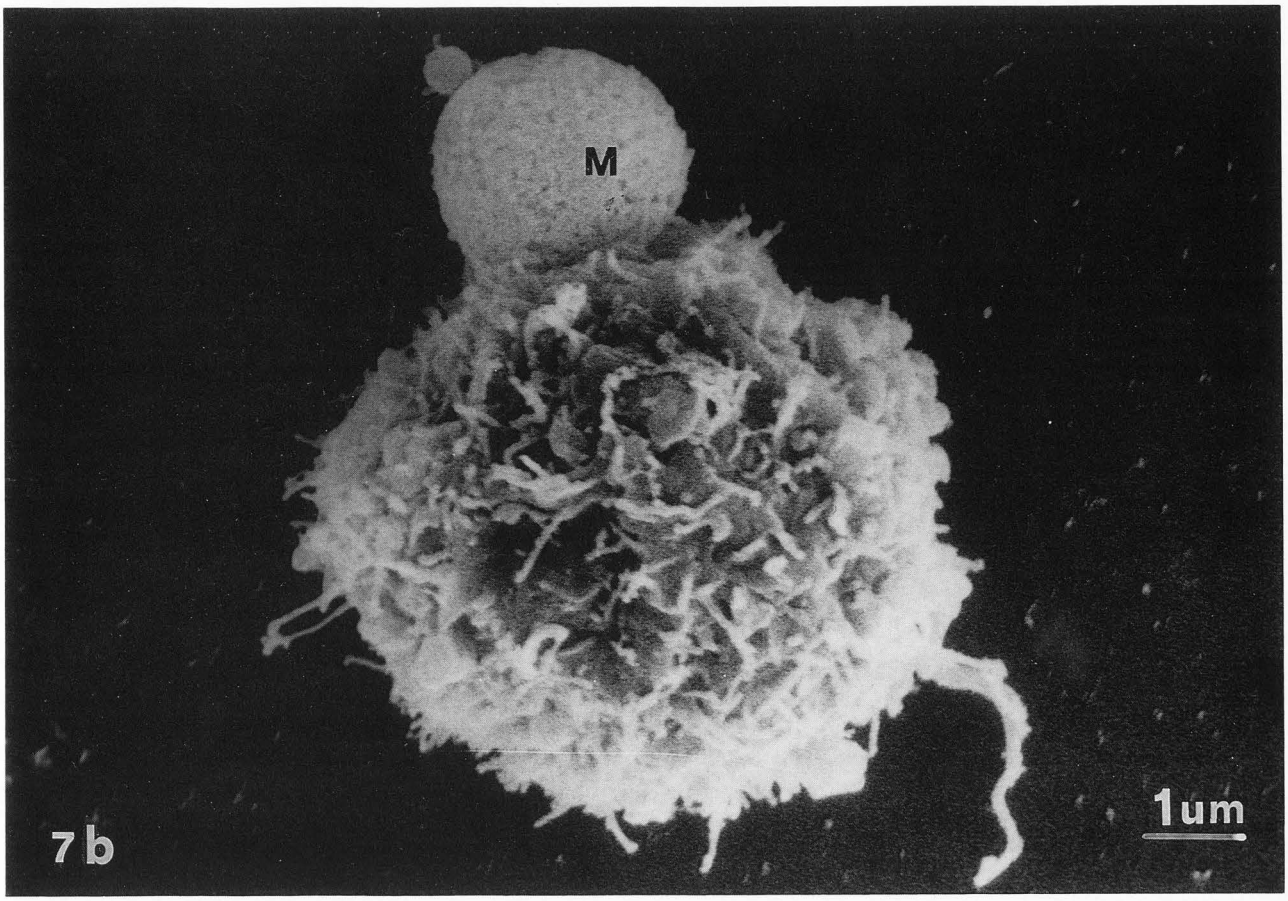

Figure 7b. Scanning electron micrographs of micronuclear extrusion from late apoptotic TF1 cells. (from Vitale *et al.*, 1993). Bar = 1 μm.

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Discussion with Reviewers

K.M. Anderson: It is possible to have no ultrastructural changes typical of apoptosis and yet obtain "DNA laddering", a hallmark of the process?

Authors: The DNA laddering has long been considered the hallmark of apoptosis. Nevertheless, some models have been described with typical apoptotic changes and no DNA cleavage (Falcieri *et al.*, 1993; Oberhammer *et al.*, 1993). Recently, Fady *et al.* (1994) have reported, that it is possible to have no morphological changes, in the presence of DNA laddering. However, some comments are necessary. Propidium iodide (PI) is a DNA-intercalating stain commonly used in flow cytometry to identify the apoptotic cell subpopulation on the base of a subdiploid peak due to the DNA loss. It normally acts after membrane permeabilization by ethanol or other solvents but we recently observed that also in fresh unfixed apoptotic cells it penetrates through the membrane during late apoptosis (Vitale *et al.*, 1993). Under certain conditions it can also be utilized as a stain specific for apoptotic cells, not only for cell lysis. We also must consider that very early apoptotic changes, like initial chromatin margination can be revealed by electron microscopy but not by PI or Hoechst staining. Finally, it is important to mention that in some models the apoptotic cell percentage is very small, so it is very difficult to identify these and determine their number.

As a general comment, we suggest that the trigger of apoptosis could be a common step which induces, depending on the different conditions, one or more of the apoptotical patterns. The molecular systems in which apoptosis takes place are in fact sufficiently different to support this hypothesis.

Only a multidisciplinary approach to the study of this phenomenon (molecular biology, genetics, flow cytometry, nick translation, electron microscopy) can clarify its mechanisms.

K.M. Anderson: Why is nucleolar structure maintained for so long in the face of extensive morphologic changes to extra-nucleolar chromatin?

Authors: The nucleolar structure can indeed be recognized for a long time during apoptosis, even if it is significantly modified and frequently shows a partial segregation of its components (Falcieri *et al.*, 1994a). Its long preservation could be correlated to the metabolic activity (RNA transcription and protein synthesis) of the cell, which is present for a long time after the induction of apoptosis and the marked chromatin changes.

J.G. Szekely: What is the time course of ultrastructural changes in apoptosis and can it be discerned from the micrographs shown?

Authors: The experimental conditions of our apoptotic models with regard to time are given in **Materials and Methods**, and have generally been reported in the papers of our group. It is difficult to draw a general conclusion on the time course of apoptosis, because the various cells, when induced to apoptosis, behave in very different manners in terms of percentage and time course.

On the contrary, what can be repeatedly demonstrated is a homogeneity of cell response at the ultrastructural level. The chromatin clustering seems in fact to be the first nuclear change, followed by the formation of cup-like condensations, characteristically marginated towards a nuclear pole.

The "*in situ* nick translation" analysis demonstrated that DNA cleavage, when present, preceded the morphological expression of apoptosis and progressively appeared in the condensing chromatin (Falcieri *et al.*, 1994a). Chromatin rearrangement also strongly affected the other nuclear components. Nuclear pores, in fact, appear to cluster all around the diffuse chromatin areas, while dense chromatin masses are pore-free, as freeze-fracture studies also indicate (Falcieri *et al.*, 1994b).

Electron dense, membrane bound micronuclei appear in subsequent stages of the process.

"Apoptotic bodies", represented by cell fragments occasionally containing nuclear material, are the final aspect of apoptosis and can be phagocytized or undergo a "secondary necrosis" process.

All these phases can be found in the same apoptotic cell population. This is due to the fact that the events triggering apoptosis are cycle-dependent and in non-synchronized cells the mechanism starts at different times. Nevertheless, at early times a few apoptotic cells can be found with initial apoptotic morphology, while later, the percentage of apoptotic cells is higher and most of these cells show micronuclei.

J.G. Szekely: Were there differences in the development of apoptosis between the different cell types? You show the examples from the various cell lines as if they are interchangeable. Is it true? I know that apoptosis is generally similar across cell types, but are there any subtle differences?

Authors: No significant differences were observed in the course of apoptosis between the different cell types. In any case, these differences seemed correlated to the morphology of the control cell (nucleo-cytoplasmic ratio; nuclear shape; chromatin condensation rate). In a previous paper (Zamai *et al.*, 1993) we described different flow cytometric patterns of apoptotic cells, dependent on cell morphology. At the ultrastructural level, only Molt 4 cells show a very early chromatin margination and a progressive formation of nuclear protrusions, probably generating micronuclei. These micronuclei are normally found scattered throughout the cytoplasm, and appear surrounded by a double membrane externally outlined by regularly distributed ribosomes. In some cases (i.e., thymocytes and TF1 cells) they are formed in the perinuclear cisterna, where they appear enveloped by a single membrane.

HL60 cells show in addition to uniformly electron dense micronuclei, also micronuclei containing diffuse or partially condensed chromatin. Molecular studies on both chromatin and nuclear envelope behavior could be useful to better investigate this phenomenon.

T.M. Seed: You state in the section **Apoptosis Versus Necrosis**: "Differently from necrosis, the nuclear organization is completely lost". Please explain!

Authors: The first target of the apoptotic mechanism is the cell nucleus. It undergoes indeed a profound rearrangement whereas the structure of the plasma membrane and the organelles are preserved for a long time. In contrast, the first target during necrosis is the plasmalemma, followed by hydration of the cytoplasm and the organelles. The nucleus maintains its organization and only in the late stages of necrosis it appears swollen and shows rearrangement of its chromatin. This image is constantly different from that of apoptosis.

T.M. Seed: Please clarify the following statement in **Molecular Events of Apoptosis**: "Mutations of these genes determine the survival of almost all cells that normally die".

Authors: *Ced-3* and *ced-4* are genes involved in apoptosis determination in *C. elegans*. It has indeed been demonstrated (Ellis *et al.*, 1991) that cells expressing *ced-3* and *ced-4* products are normally committed to apoptosis. In contrast, when a mutation of these genes has occurred, these cells survive.