ORIGINAL PAPER

Eur. J. Histochem.
44: 237-246, 2000

Cell shape and organelle modification in apoptotic U937 cells

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Accepted:

Key words: U937, apoptosis, blebbing, nuclear fragmentation, mitochondria

SUMMARY

U937 cells induced to apoptosis, progressively and dramatically modified their cell shape by intense blebbing formation, leading to the production of apoptotic bodies. The blebs evolved with time; milder forms of blebbing involving only a region or just the cortical part of the cytoplasm were observed within the first hour of incubation with puromycin; blebbing involving the whole cell body with very deep constrictions is the most frequent event observed during late times of incubation. The ultrastructural analysis of apoptotic cells revealed characteristic features of nuclear fragmentation (budding and cleavage mode) and cytoplasmatic modifications. The cytoplasm of blebs does not contain organelles, such as ribosomes or mitochondria. Scarce presence of endoplasmic reticulum can be observed at the site of bleb detachment. However, blebbing is a dispensable event as evaluated by using inhibitor of actin polymerization.

In the present study, the progressive modifications of the nucleus, mitochondria, nuclear fragmentation, cytoplasmic blebs formation and production of apoptotic bodies in U937 monocytic cells induced to apoptosis by puromycin (an inhibitor of protein synthesis) were simultaneously analyzed.

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INTRODUCTION

Apoptosis, a physiological mode, is a self-directed process of cell death that proceeds with characteristic biochemical and cytological features, including DNA digestion into high (300 and 50kpb) and low (ladder of multiple of 200 bp) molecular weight fragments (Zhang *et al.* 1998; Khodarev *et al.* 1998). In parallel with the biochemical processes, many morphological modifications become progressively visible. Morphological changes include cell shrinkage, chromatin condensation at the nuclear periphery, and nuclear fragmentation in several membrane-bound vesicles (Kerr 1971; Falcieri *et al.* 1994; Earnshaw 1995; Kass *et al.* 1996; Saraste 1999; Robertson *et al.* 2000) and the formation of actin-dependent (Lemaster *et al.* 1983; Huot *et al.* 1998; Asumendi *et al.* 2000; Spano *et al.* 2000) cytoplasmic protrusions (blebs) which eventually detach from the cell, thus forming the so-called "apoptotic bodies" (Mills *et al.* 1998; Gores *et al.* 1990). Apoptotic blebbing is characteristic of many, but not every, cell and requires major cytoskeletal reorganization; it has been related to Ca^{++} and thiol disturbances, which may interfere with the actin cytoskeletal network (Nieminen *et al.* 1988; Fishkink *et al.* 1991; Cunningham 1995; Martin *et al.*

1995; Miyoshi *et al.* 1996; Atencia *et al.* 2000). Inhibition of actin polymerization inhibits blebbing without affecting the development of other apoptotic features (Huot *et al.* 1998).

The process of apoptosis is very rapid, and intermediate stages are not easily detectable. The end point of this process is the rapid removal of the apoptotic cells and/or bodies by phagocytosis, a process involving membrane changes which stimulate and address a correct phagocytic recognition (Fadok *et al.* 1998a; Dini, 2000). Indeed, cell death by apoptosis does not elicit an inflammatory response since the uptake of the dead cells occurs before any leakage of intracellular material (Fadok *et al.* 1998b). The ultimate fate of the internalized dead cells is their definitive digestion inside phagosomes (Savill 1998; Wu and Horvitz 1998). To this purpose, nuclear and cytoplasmic fragmentation, beside DNA digestion, may have a physiological meaning in promoting the phagosome's digestion, and may not only be a series of catastrophic events. Therefore, cell surface modifications and cell and nuclear fragmentation are essential events for the 'apoptotic bodies' rapid removal and subsequent digestion by means of a non inflammatory and non-immunogenic elimination (Meagher *et al.* 1992; Ren *et al.* 1998).

In this study, we simultaneously analyze the progressive modifications of the nucleus, mitochondria, nuclear fragmentation, cytoplasmic blebs formation and production of apoptotic bodies in U937 cells induced to apoptosis by puromycin, an inhibitor of protein synthesis.

MATERIALS AND METHODS

Cells and Treatments

Cells and cultures: U937 cells were kept in a log phase in RPMI 1640 medium (Mascia Brunelli - Italy) supplemented with 10% inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin in a controlled atmosphere (5% CO2) incubator at 37°C. Experiments were performed at a concentration of 10^6 cells/ml.

Induction of apoptosis: Apoptosis was induced with 10 μg/ml puromycin (PMC).

Analysis of Apoptosis and analysis of blebbing

Apoptosis was detected by light and electron microscopy and by cytofluorimetry. Nuclear fragmentation was detected after staining with the vital dye Hoechst 33342 (1μg/ml) according to the nuclear morphological feature.

Ultrastructure of apoptotic cells was obtained by Trasmission and Scanning electron microscopy. 10^6 cells/ml were fixed with 2.5% glutaraldehyde in cacodilate buffer, pH 7.4, for 1h at ice temperature, postfixed with 1% OsO4 in the same buffer, dehydrated, embedded in Spurr resin and examined under a Philips CM12 TEM. SEM observation was done on U937 deposited on poly-L-lysine-treated coverslip slides. Critical Point Dryer 020 Balzer and Sputter Coated 040 Balzer were used for the final preparation steps. Cells were examined under a Philips XL50 scanning microscope.

Quantitation of Apoptosis: The quantification of apoptotic cells was performed by flow cytometry and by light microscopy morphology.

Percentage of apoptotic and viable cell fractions was performed by cytofluorimetry. EPICS XL cytofluorimeter (Coulter Electronic Inc. Hialegh FL) with 1024 channel of resolution was used with propidium iodide (10 μg/ml) labelled U937.

The fraction of cells with fragmented, crescent-shaped, or shrunken nuclei was evaluated among the Hoechst-stained cells by counting at least 300 cells in at least three randomly selected fields at light microscopy. Cell viability was assessed by normal nuclear shape and texture revealed upon vital staining with Hoechst 33342.

Inhibition of cell blebbing: To inhibit the formation of membrane blebs cytochalasin B (CCB) (5μg/ml) were added to culture medium 30 min before the apoptogenic treatment and maintained throughout incubation.

Ca^{++} localization: The intracellular Ca^{++} (membrane bound) localization was visualized by means of the yellow-orange fluorescence due to chlorotetracycline (CTC)/Ca^{++} complex (Tretyn and Kopcewicz 1988). U937 were fixed with 4% paraformaldehyde in 0.1M PBS pH 7.2 at room temperature on glass slides and incubated with 10^{-3} CTC in the same buffer for 30 min in the dark. Mounted slides were observed with a Zeiss Axioskop light fluorescence microscope.

RESULTS

We followed cell shape and organelle modifications in U937 monocytic cells induced to apoptosis by puromycin, a protein synthesis inhibitor that

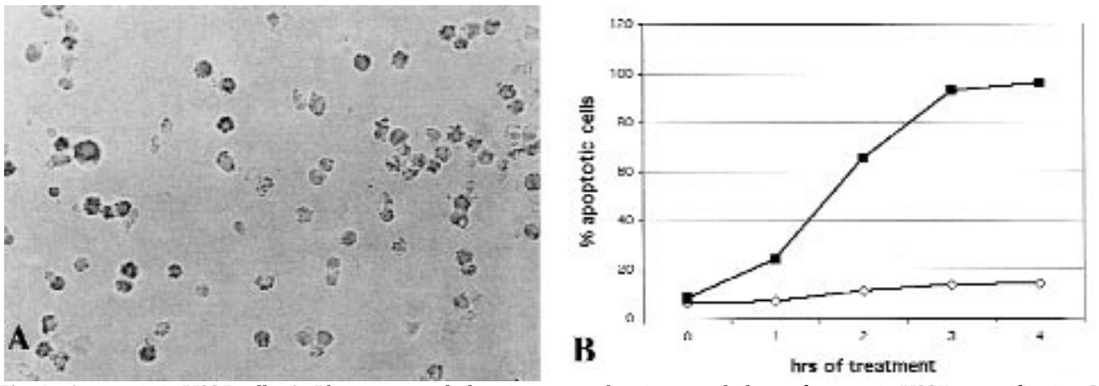


Fig. 1 - Apoptosis in U937 cells. A: Phase contrast light microscopy showing morphology of apoptotic U937; magnification 600x; B: Time course of induction, quantified by flow cytometry as described in material and methods. One experiment among 5 is shown.

produces almost 100% apoptotic cells in four hours of continuous incubation. The percentage of necrosis in the fraction of dead cells is minimal (about 3%) (Fig. 1). Apoptotic U937 cells always have fragmented nuclei and DNA is digested in the characteristic ladder-like pattern.

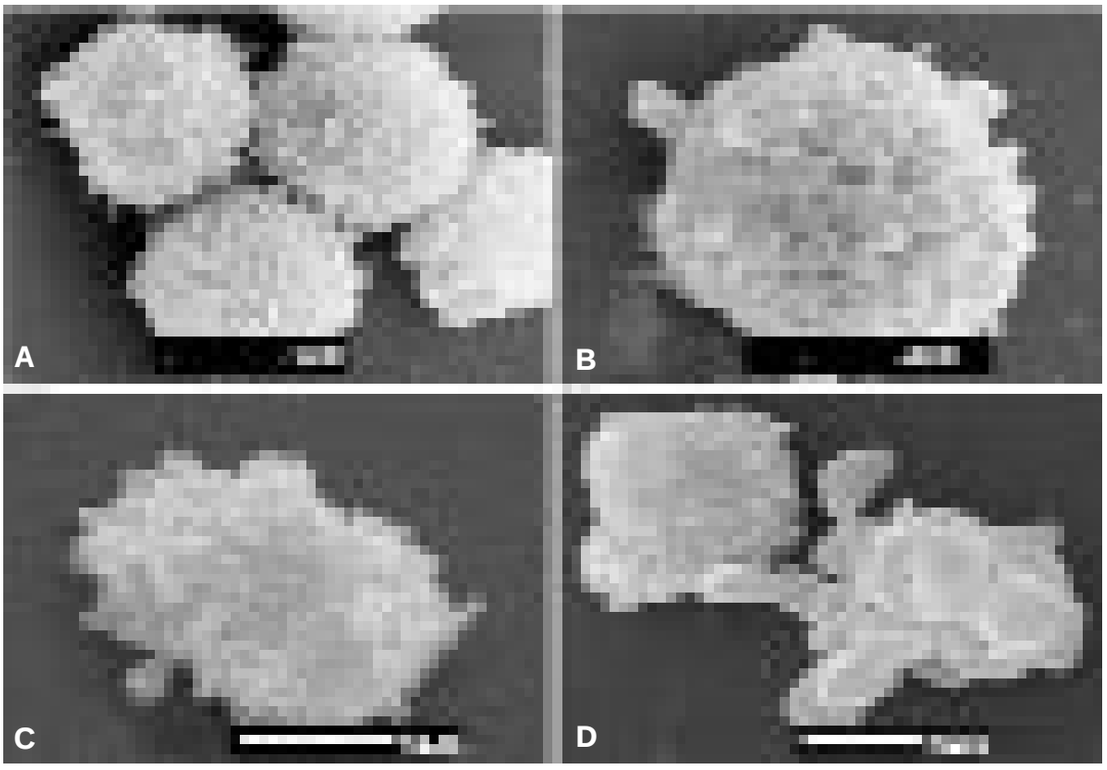


Fig. 2 - Time course of blebbing in apoptotic U937. SEM micrographs showing the progressive loss of cell shape and the progressive formation of blebs. A: normal U937 showing rough surface with small pseudopodia; B: after 1hour of incubation with puromycin, the pseudopodia are changing and are becoming more spherical, shaped; C: after 2.5 hours, the cells are starting to stretch and D: after 4 hours of continuous incubation with the drug, numerous blebs are forming, prone to detach from the cell body.

The cell shape was directely analyzed by observation of living cells at the phase contrast microscope and on fixed cells at light and electron microscopy (TEM and SEM). Cell shape alterations and blebbing are strikingly rapid phenomena: the irregular round shape of the living cells with many pseudopodia-like protrusions is replaced by the smooth round shape of apoptotic cells protruding spherically shaped blebs. The presence and distribution of blebs in the U937 cells is time dependent (Fig. 2). Early modifications include the presence of milder forms of blebbing which involve only a region or just the cortical part of the cytoplasm. With increasing time, blebbing involves the whole cell body with very deep constrictions and the original cell shape is entirely destroyed (Fig. 2). Since blebbing has been related to Ca^{++} and thiol disturbance, which may interfere with the actin cytoskeletal network (Nieminen *et al.* 1988; Fishkink *et al.* 1991; Cunningham 1995; Martin *et al.* 1995; Miyoshi *et al.* 1996), membrane bound Ca^{++} has been detected by labelling with CTC (Canini *et al.* 1993). Ca^{++} surrounds the nucleus in normal living cells and, upon blebbing, is redistributed inside the cytoplasm. Blebs are always negative to the labelling (Fig. 3).

By TEM ultrastructure analysis, changes occuring in the cytoplasm and organelles during blebbing were monitored. Two modes of nuclear fragmentation characterized the nuclear modifications: budding and cleavage (Fig. 4). The evolution of the morphological changes involve chromatin condensation at the nuclear periphery, forming patches protruding from the nuclear envelope, which evolve into buds leading to nuclear fragmentation. In an alternative process, chromatin condenses in tiny, regularly shaped crescents involving most of the nuclear edge, without protruding. The subsequent fragmentation occurs by cleft in the nuclear sap at a point in the nuclear periphery which is free of condensed chromatin (Dini *et al.* 1996). However, irrespective of the modality of fragmentation, mature/late apoptotic U937 cells present a total nuclear fragmentation in different sized fragments, occasionally visible also inside blebs. Interestingly, a deep nuclear constrinction is always related to strongly blebbing cells (Fig. 5). Cell blebbing is a dispensable event in apoptosis. CCB completely eliminates cell blebbing and the resulting cellular fragmentation; however, apoptosis is still morphologically recognizable, since chromatin condensed at the nuclear periphery is easily detectable (Fig. 6).

In the majority of blebs, the cytoplasm does not contain organelles, such as ribosomes or mitochondria. Sometimes, a scarce presence of endoplasmic reticulum can be observed at the site of the bleb detachment, forming a regular line of small vesicles. Morphologically intact mitochondria are observed during the early phases of apoptosis. Their position

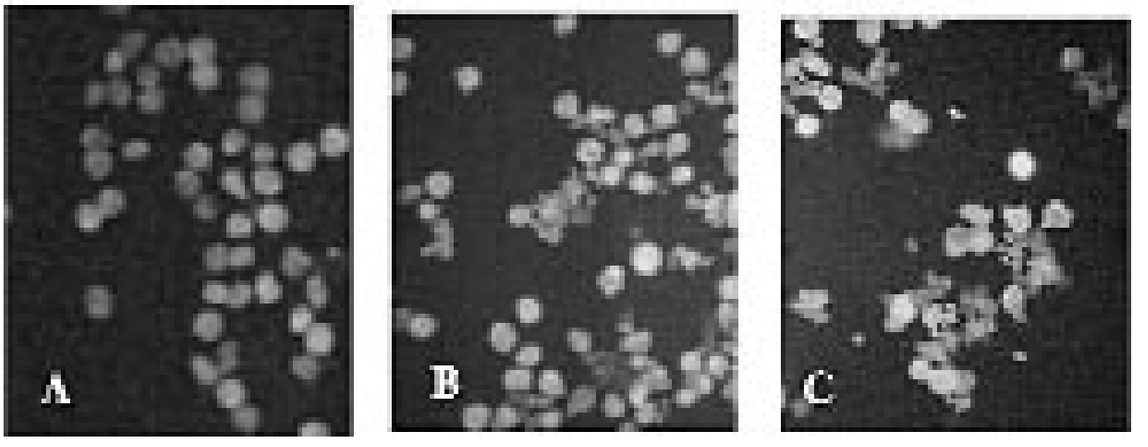


Fig. 3 - Chlorotetracycline labelling in apoptotic U937 cells. Fluorescent light micrographs showing the presence of membrane bound Ca^{++} in (A) normal cells and (B,C) after puromycin incubation. In living cells the fluorescence is concentrated around nuclei, while the periphery of cell is scantily fluorescent. Arrows indicate the absence of the fluorescent dye in the blebs of apoptotic U937 cells. Magnifications: 1000x.

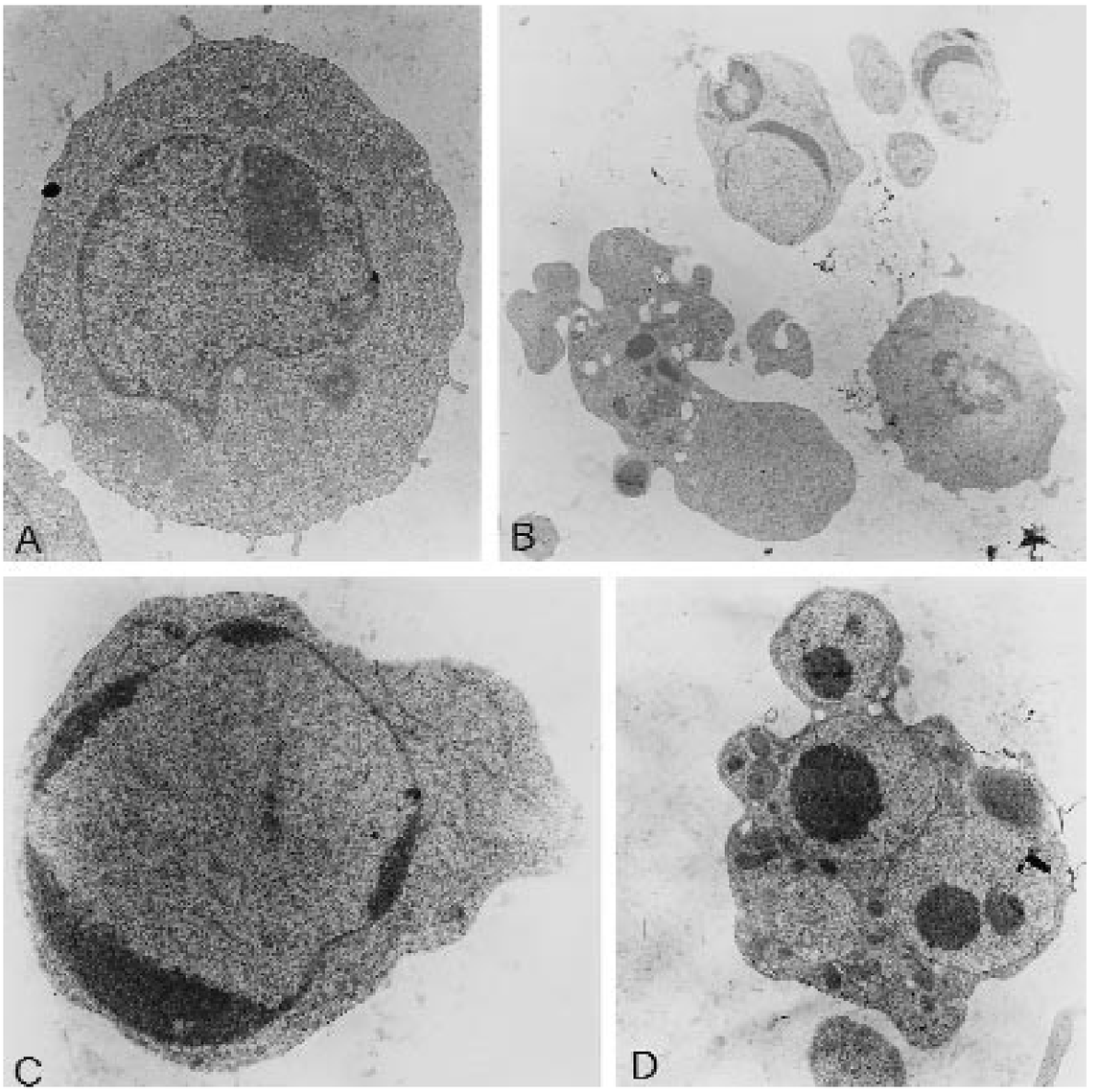


Fig 4 - Representative apoptotic nuclear morphologies. TEM micrographs showing the different nuclear morphologies observed during induction of apoptosis in U937 by puromycin. A: normal cells with beam shaped nucleus, nucleolus, and condensed and non condensed zones of chromatin; B: Tiny crescents of condensed chromatin that will fragment by cleavage; C: Patches of condensed chromatin along the inner nuclear envelope that will fragment by budding; D: mature/late apoptotic U937 with completely fragmented nucleus. Magnifications: 8200x (A); 4200x (B); 13000x (C); 8200x (D).

inside the cytoplasm is affected by blebbing. During the cell shape changes, they are squeezed from their normal position and concentrate near the nucleus and far from the blebs (Fig. 7). In fact, mitochondria localization inside the blebs is unfrequent. During this migration, the morphology of mitochondria remain unchanged. However, at the ultrastructural level, mitochondria disappear in the late apoptotic U937 cells. The reason for the reduction of morphologically intact mithocondria and, as a consequence, loss of function, must be found in their important role during the apoptotic process. To support this

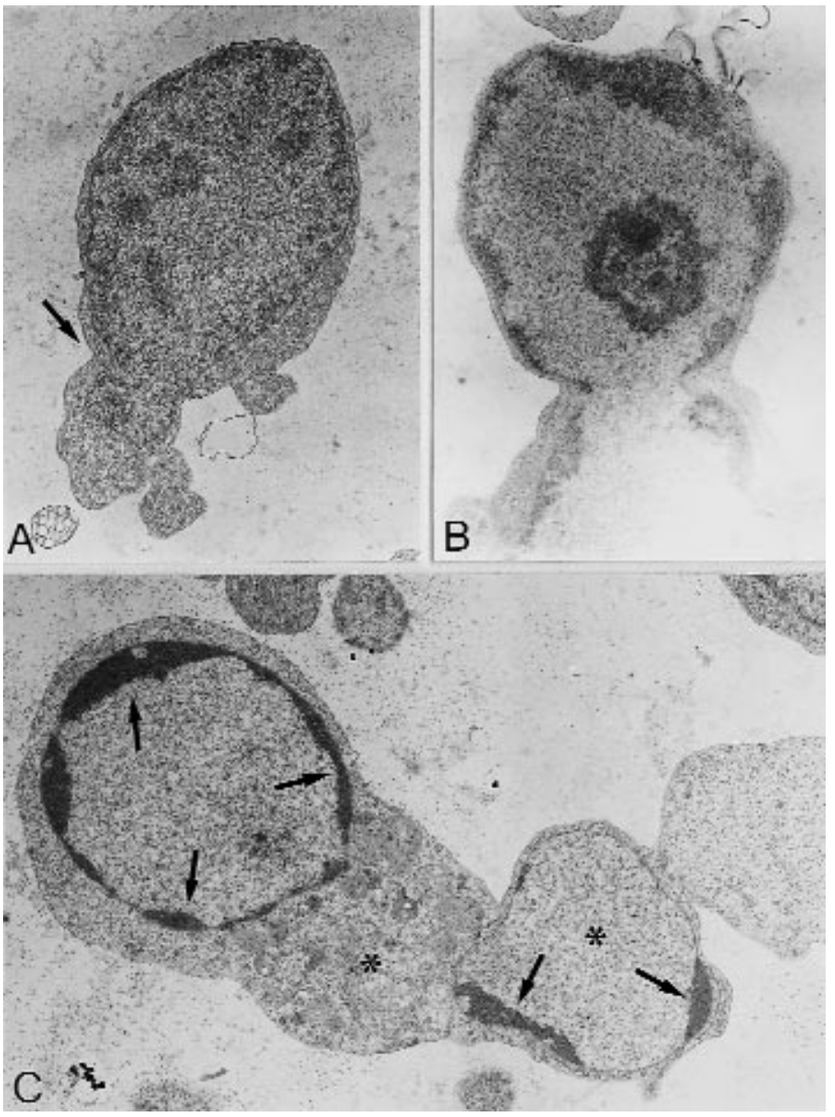
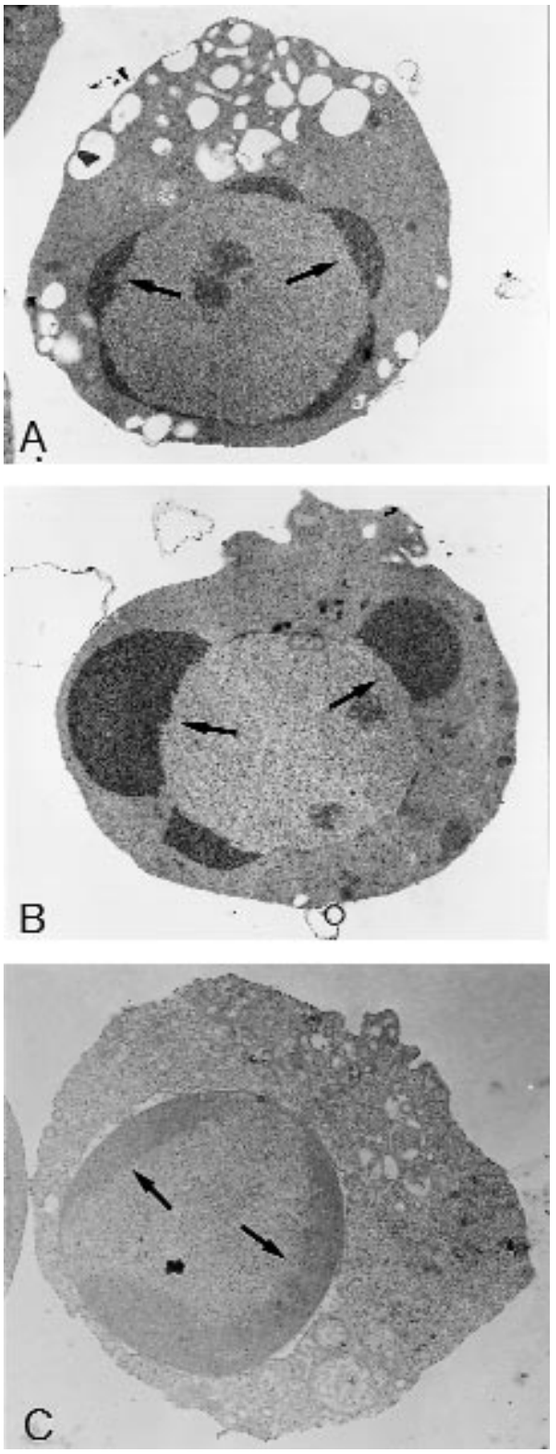


Fig. 5 - Blebbing and nuclear constriction in apoptotic U937 cells. TEM micrographs showing the progressive nuclear fragmentation induced by blebbing in apoptotic U937 cells. A: initial nuclear constriction (arrow) due to the first apperance of a bleb. Chromatin is just starting to condensate; B: particular of a nuclear constriction following the production of a bleb. Condensate chromatin is present; C: strongly blebbing cells develop deep nuclear constrinctions and subsequent fragmentation (asterisks). Condensed chromatin (arrows). Magnifications: 7000x (A); 11200x (B); 8000x (C).

finding, an increasing number of degenerating mitochondria, or only vesicles showing a double membrane, are observed during late apoptosis (Fig. 7).

DISCUSSION

The time course modifications of some typical features of apoptosis (i.e. cell shrinkage, dynamic membrane blebbing, condensation of chromatin and nuclear fragmentation) have been described in the present work. The evolutionarly conserved execution phase of apoptosis is defined by the above-mentioned characteristic changes occurring during the final stages of death. However, the description of these hallmark features of apoptosis has been previously elusive, largely because the execution phase is a rapid event whose onset is asynchronous across a population of cells. By means of electron microscopy, progressive ultrastructural changes can be described in the apoptotic cell, as well as the appearance of its typical

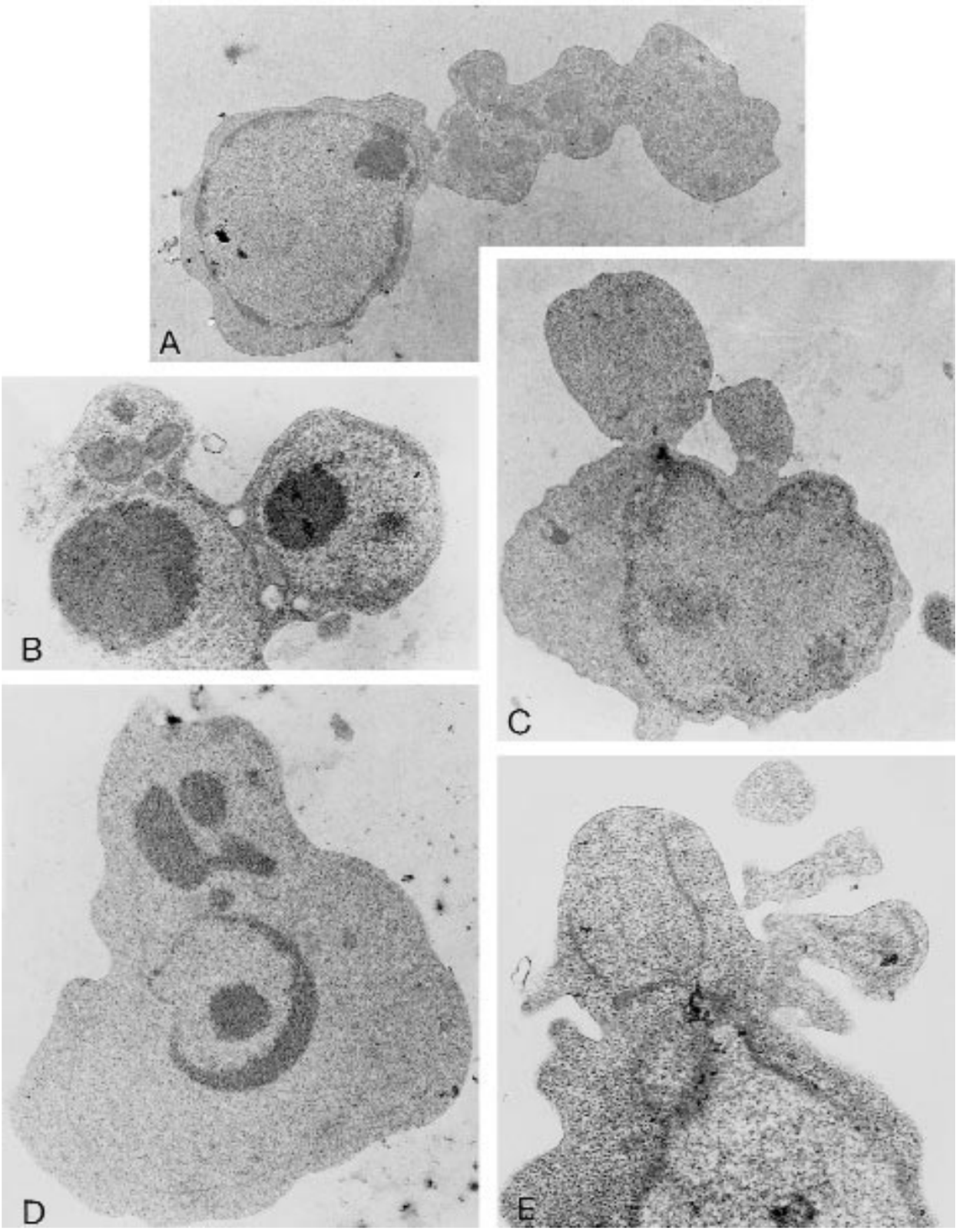


aspects (Falcieri *et al.* 1994; Saraste 1999; Moon *et al.* 2000). Morphology represents the phenotypical expression of the biochemical and/or molecular changes occuring to the cells during apoptosis. The different apoptotic morphologies that have been so far described are a picture of each single step of the biochemical pathway chosen by the cell during its progression in the apoptotic program (Ghibelli *et al.* 1995). This is in agreement with the involvement of various metabolic pathways (membrane or nuclear receptors, oxidative stress, mitochondria, etc.) in the apoptotic induction (Saini and Walker 1998).

Two main, cell-specific types of nuclear shape alterations have been reported to accompany chromatin condensation in apoptotic cells: mild (i.e., lymphocytes, thymocytes) or strong (i.e., Jurkat cells) nuclear shrinkage, or, in those cell types where shrinkage is negligible or absent, nuclear fragmentation (Nagata 2000). Nuclear fragmentation can be obtained by multiple choice (Dini *et al.* 1996), thus suggesting evolutionary convergence. Therefore, the nuclear fragmentation (micronuclei formation) and apoptotic bodies formation may be crucial events in the death program, whose importance may deal with the clearance of apoptotic bodies, since nuclear fragmentation is a late and possibly dispensable step in apoptosis.

Blebbing is a good marker of apoptosis independently of the apoptogenic stimulus; it has been found associated with apoptotic ladder formation and nuclear fragmentation (Takano *et al.* 1991). Apoptotic blebbing requires actin polymerization as demonstrated by our and other's experimental data (Mills *et al.* 1998). F-actin has been suggested by Asumendi and coworkers (2000) to be correlated to the characteristic shape observed in apoptosis. Additionally, the disposition of G-actin

Fig. 6 - Inhibition of cell blebbing and nuclear fragmentation. Cytochalasin B 5µg/ml inhibited nuclear fragmentation, whereas chromatin condensation is not (arrow); different progression of the chromatin condensation at the nuclear edge is easily recognizable: A) early condensation of chromatin at the nuclear periphery; B) CCB inhibits the formation of nuclear vesicles, and the patches of condensed chromatin are very protruding. Nucleoli are still visible; C) early signs of secondary necrosis in apoptotic U937 cells without any signs of blebbing and fragmentation. The two nuclear membranes fall apart where condensed chromatin has accumulated. Magnifications: 6000x (A); 7500 (B); 7500x (C).



could have a relationship with the cleavage of DNA (Asumendi *et al.* 2000). In conditions in which actin cannot be modified, thus being free to dangerously polymerize without restraint, abnormal cellular fragmentation is described (Ghibelli *et al.* 1995). The peculiar distribution of the cell organelles inside blebs could be also ascribed to the cytoskeleton rearrangement. The physiological reason of this asymmetry is still unknown.

Previous studies have shown that changes in mitochondria, such as decreased mitochondrial potential and production of reactive oxygen species, can also occur in apoptosis and precede other apoptotic event (Vayssiere *et al.* 1994; Zamzami *et al.* 1995). However, morphological and functional changes in mitochondria have been described only in a few models of apoptosis. Our data showing a disappearance of morphologically intact mitochondria are in agreement with the work showing a decrease in mitochondrial potential relative to mitochondrial mass, thus suggesting that an accumulation of damaged or dysfunctional mitochondria occurred (Camilleri-Broet *et al.* 1998).

In the analysis of apoptotic morphologies described in this study, we show a relationship among different typical features of apoptosis such as nuclear fragmentation, plasma membrane blebbing, Ca^{++} distribution, thus suggesting a close correlation between cytoplasmic and nuclear parameters of apoptosis.

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Fig. 7 - Ultrastructure of blebs in apoptotic U937 cells. TEM micrographs showing diverse aspects of blebs. Generally, the cytoplasm of blebs is free of ribosomes, reticulum, mitochondria, etc. (C, D). Rarely, endoplasmic reticulum forms a regular line of small vesicles at the site of the bleb detachment (B, E). Morphologically intact mitochondria are observed squeezed from their normal position and concentrate near the nucleus and far from the blebs (A). However, at the ultrastructural level, mitochondria disappear in the late apoptotic U937 cells. Micronuclei (sometimes with distinguishable nucleoli) are very often seen inside the blebs, their natural site before their extrusion from the cell (B,D; B is a particular at higher magnification of Fig. 4D). Magnifications: 6000x (A); 15500x (B); 7000x (C); 8800x (D); 26500x (E).

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