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Synchronized onset of nuclear and cell surface modifications in U937 cells during apoptosis

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SUMMARY

In this study we investigated the relationship between nuclear and cell surface modifications (i.e. blebbing, phosphatidylserine [PS] and sugar residues exposure) in a monocytic cell line, U937, during apoptosis induced by oxidative stress (1mM H_2O_2) or inhibition of protein synthesis (10 μg/ml puromycin). Dying cells were simultaneously observed for nuclear modifications, presence of superficial blebs and plasma membrane alterations.

Morphological analysis performed by conventional fluorescence microscopy, or by transmission and scanning electron microscopy showed that the courses of nuclear and membrane alterations occured concomitantly, but the phenotype was dependent on the stage of the apoptotic process and the type of apoptogenic inducer used. The progression of apoptosis in U937 cells beyond early stages resulted in the extensive formation of blebs which concomitantly lost some typical markers of apoptosis, such as PS and sugar residues. Therefore, the modality by which the nucleus condenses, or the amount and the pattern of distribution of PS on the cell surface were, for each cell line, strictly related to the apoptogenic inducer.

The morphological data reported in the present paper should lead to a more precise quantification of apoptosis by improving the detection of apoptotic cells *in vivo* (i.e. in tissue, organs), which is a crucial point in the evaluation of efficiency of antiproliferative drugs, such as antiblastic or immunosuppressive compounds.

INTRODUCTION

Many morphological changes, such as cell shrinkage, cytoplasm and chromatin condensation, characterize apoptosis. This subsequently leads to the fragmentation of cells into clusters of membrane-bound structures or apoptotic granular bodies in which the integrity of various subcellular organelles is initially maintained (Wyllie *et al.* 1980). The apoptotic bodies are incorporated into phagocytes or neighboring cells and the DNA breaks up at the internucleosomal spaces into oligome fragments (Nagata, 2000; Dini, 2000; Savill, 1998; Platt *et al.* 1998; Fadok and Henson, 1998; Dini, *et al.* 1996).

Development of the characteristic apoptotic morphological features depends on the specific bio-

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chemical events involved in the process of apoptosis induced by different stimuli (Dini *et al.* 1996; Kumar and Harvey 1995; Falcieri *et al.* 1994a). In fact, morphological modifications occurring in the nucleus of cells dying by apoptosis are related to phase of apoptotic progression and the apoptogenic inducer used (Dini *et al.* 1996).

A peculiar feature of apoptosis is the nuclear fragmentation that follows DNA fragmentation and chromatin condensation. Two principal forms of nuclear fragmentation have been described to occur in U937 cells (Dini *et al.* 1996). The most common modality is fragmentation by budding which consists in the condensation of chromatin to form large clumps that press against the nuclear envelope and protrude from the nuclear membrane. The protrusion evolves in budding with consequent detachment of membrane-bound nuclear fragments. However, in an alternative process occurring within the same cell, chromatin condenses in tiny, regularly shaped crescents involving most of the nuclear border, without protrusion. The successive fragmentation occurs by a cleft in the nuclear sap at a point in the nuclear periphery which is free of condensed chromatin (Dini *et al.* 1996).

Moreover, during the apoptotic process, dynamic modifications occur in plasma membrane structures and on the cell surface (Mills *et al.* 1998; Rudel and Bocock 1997). Dying cells are often blebbing dramatically such that the cell structure is completely modified. Many blebs extrude from the cytoplasm giving to cell a foamy shape. The blebs can detach from the cell and generate "apoptotic bodies". The mechanism(s) involved in blebs formation is unknown. However, redox perturbation and reassembling of actin filaments favour and/or control cellular blebbing (Wyllie *et al.* 1980).

The apoptotic process is also characterized by peculiar cell surface modifications such as expression of specific molecules (antigens and/or receptors) or alteration in lipid asymmetry and carbohydrate structure (Fadok and Henson 1998; Dini *et al.* 1992; Morris *et al.* 1984). These modifications have great importance during the recognition and removal of apoptotic cells by phagocytes (Savill 1998). It has been difficult up to now to identify changes likely occurring at the cell surface during the early phases of apoptosis while the cell continued to appear overtly normal and healthy, but which were responsible for their swift removal. It is important to note that the above studies do not describe the evolution of "apoptotic phenomena", since they only describe morphological features corresponding to a biochemical step of the apoptotic machinery. Therefore, in the present work, the time course of morphological modifications of U937 cells induced to apoptosis by different apoptogenic inducers (oxidative stress, protein synthesis inhibition) has been studied. Dying cells were simultaneously observed for their nuclear modifications, presence of superficial blebs and plasma membrane alterations.

MATERIALS AND METHODS

Cells and Treatments

Cells and cultures
U937 cells were kept in a log phase in RPMI 1640 medium (Mascia Brunelli, Milano, Italy) supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin in a controlled atmosphere (5% CO_2) incubator at 37°C. Cells were used with a concentration of 10^6 cells/ml.

Induction of apoptosis
Apoptosis was induced with 1mM freshly prepared hydrogen peroxide (H_2O_2) for 1hr, followed by 1, 3 and 4 hrs recovery in fresh medium; or induced with 10 µg/ml puromycin (PMC) for 1,3 and 4 hrs.

Analysis of apoptosis and evaluation of cell shape
Apoptosis was detected by light and electron microscopy and by cytofluorometry. Nuclear fragmentation was detected after staining the cells with the vital dye Hoechst 33258 (1g/ml) according to the nuclear morphological feature.

Ultrastructure of apoptotic cells was obtained by trasmission and scanning electron microscopy (TEM and SEM, respectively). 10^6 cells/ml were fixed with 2,5 % glutaraldehyde in cacodylate buffer, pH 7.4, for 1h at ice temperature; postfixed with 1% OsO_4 in the same buffer, dehydrated, embedded in Spurr resin and examined under a Philips CM12 TEM. U937 cells deposited on poly-L-Lysine treated coverslip slides were observed at SEM. Critical Point Dryer 020 Balzer and Sputter

Coated 040 Balzer were used for the final preparation steps. Cells were examined with a Philips XL50 SEM.

Quantitation of Apoptosis

The percentage of apoptotic and viable cell fractions was quantited by cytofluorimetry. An EPICS XL cytofluorimeter (Coulter Electronic Inc. Hialeah, FL) with 1024 channels of resolution was used with propidium iodide (10g/ml) stained U937 cells.

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 (10 min in the dark) with Hoechst 33342.

The type of nuclear fragmentation of dead cells was analyzed using the dye, Hoechst 33258, on fixed cells laid on gelatinized slides, and by analyzing its ultrastructure on glutaraldehyde-fixed cells. The visualization of nucleus and the condensation status of the chromatin was observed, respectively, at the fluorescence microscope and at TEM.

Cell Surface Modifications

Structural modifications of the cell surface were examined by SEM and lectin cytochemistry (conventional and confocal microscopy and cytofluorometry analysis).

Conventional and confocal microscopy

Apoptotic U937 cells, fixed with 4% paraformaldehyde in phosphate buffer pH 7.4 for 10 min., and deposited on gelatinated microscopy slides were analyzed for surface localization of sugars by using FITC-labeled conjugates with different specificities. The following lectins were used (in brackets are reported the concentrations, the abbreviations and the sugar specificity): *Helix pomatia* (HPA, 60μg/ml, N-acetylgalactosamine); *Ulex europaeus* (UEA, 40 μg/ml, α-fucose); *Phytolacca americana* (PWM, 60μg/ml, N-acetylglucosammine/galactose); *Concanavalin-A* (Con-A, 40 μg/ml, mannose); *Triticum vulgaris* (WGA, 120μg/ml, N-acetylglucosamine, neuraminic Acid). All lectins were from Sigma (St. Louis, MO, USA). Annexin V-FITC (Genzyme, Cambridge) (6 μg/ml for 30 min in the dark) was used for detection of phosphatidylserine (PS) on the surface on dead cells.

Samples were observed with conventional and confocal fluorescent microscopy. Images were acquired by means of a Biorad MRC-1024 confocal microscope, using a 60x/1.4 NA immersion oil lens. 3D stack were acquired at resolution of 0.1 micron in X, Y and Z axis. 3D image processing (image deconvolution) was applied in order to increase planar and axial resolution (by public domain software: XCOSM from Washington University).

Flow cytometry

Quantitation of binding of lectins and Annexin V-FITC conjugates to U937 cells were evaluated by cytofluorimetry. U937 cells were incubated with 10 µg/ml of the fluorescent lectins for 20 min.or with 0.5 µg/ml of Annexin V-FITC for 10 min. at 4°C in the dark.

RESULTS

Quantitation of apoptosis and nuclear modifications

Two substances (puromycin and hydrogen peroxide) with different cellular targets induced U937 cells to apoptosis. The apoptotic indexes were measured by flow cytometry at different times of incubation or recovery (within 4 hrs) (Fig. 1). The apoptotic rate was dependent on the apoptogenic stimulus; it was higher in puromycin-induced cells versus H_2O_2-treated U937 cells. Puromycin induced (at the concentration of 10 µg/ml) more than 80% of apoptosis in U937 cells within 4 hrs versus 40% of apoptosis in H_2O_2-treated U937 cells. But, as a consequence of the oxidative stress, the apoptotic peak was reached more rapidly (3 hrs) than it was after inhibition of protein synthesis (4 hrs).

The type of nuclear fragmentation of dead cells was analyzed by fluorescence microscope and TEM. From these observations, nuclear fragmentation by budding or cleavage, total fragmentation, mitosis/apoptosis, chromatin condensation and nuclear "constriction" without chromatin condensation were simultaneously observed in both treatments (Fig. 2). However, within the same treatment (i.e. H_2O_2 or puromycin), the frequency of each morphology was different. The most frequent nuclear fragmentation for H_2O_2 treated cells is by budding, while nuclear fragmentation by cleavage is the process most fre-
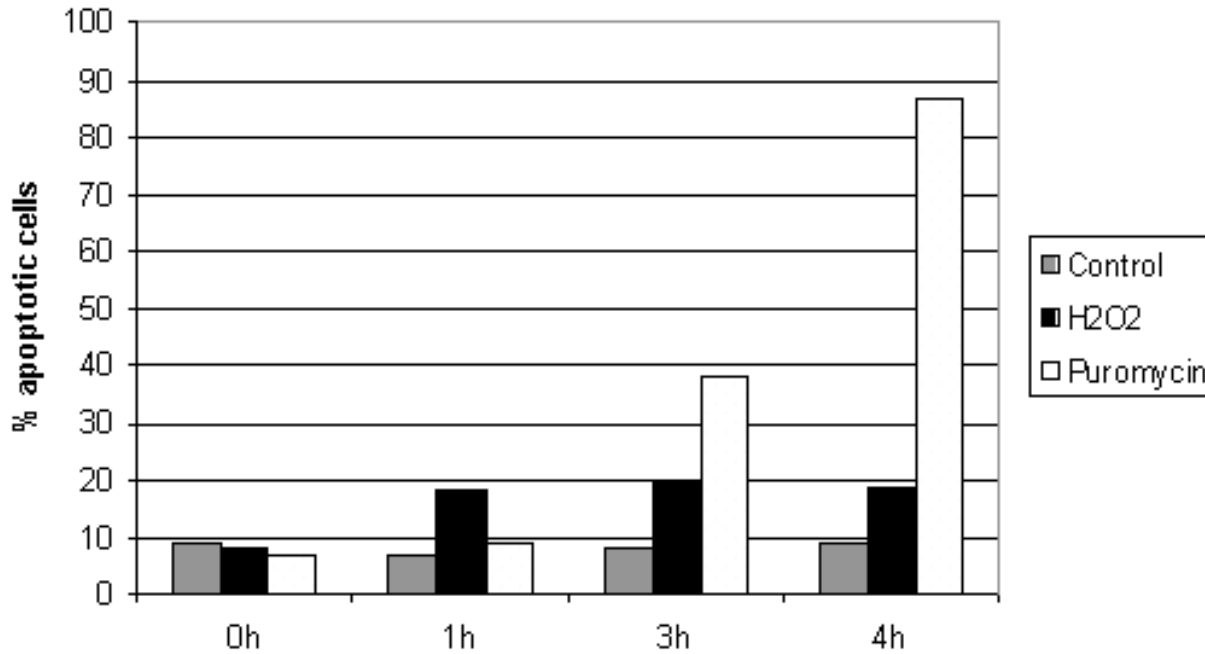
Fig. 1 - Time course of apoptosis in untreated U937 cells or cells treated with H_2O_2 and puromycin. The values were obtained by flow cytometry of P.I.-labelled cells during 4 hrs of recovery after 1hr of oxidative stress (1mM H_2O_2) or during 4 hrs of incubation with puromycin (10µg/ml). After 4 hrs of incubation, at least 80% of U937 were apoptotic. 1 hr of 1mM H_2O_2 induced 50% of apoptosis at 3hrs of recovery.

Table I
Summarizes the main features observed during the apoptotic processes in U937 cell lines

	APOPTOSIS H_2O_2 induced				APOPTOSIS Puromyci n induced		
	EARLY	MATURE	LATE		EARLY	MATURE	LATE
NUCLEUS	budding	budding	total fragmentation	NUCLEUS	cleavage	cleavage	total fragmentation
PS	+ +	- - (*)	- - - (*)	PS	+	- - (*)	- - - (*)
SUGARS	-	- - (**)	- - - (**)	SUGARS	-	- -	- - -
BLEBS	-	-	-	BLEBS	+/ -	+	+ +

+ increase; - decrease. (*)The level of PS decreased as compared to the early phases, but was always higher than control. (**) The level of sugar residues was always lower than control.

Fig. 2 - Electron micrographs of main apoptotic nuclear morphology of U937 cells. The nucleus of a normal (a) U937 cell is bean-shaped with clods of uncondensed chromatin. Two principal ways of nuclear fragmentation are observed in apoptotic U937 cells: budding and cleavage. The early phase during progression of apoptosis can be seen either in (b) (budding) or in (c) (cleavage). In the budding modality, condensing chromatin forms large clumps which press against the nuclear envelope, bud and detach from the nuclear membrane. In an alterntive process, chromatin condenses in nonprotruding crescents. A cleft in the nuclear sap forms at a point in the inner nuclear membrane, which remains close to the nucleoplasm, whereas the outer membrane does not follow the cleavage. Total nuclear fragmentation is shown in (d) while in (e) the nucleus is squeezed by modifications of the cell shape without condensation of the chromatin. A small percentage (about 5%) of apoptotic cells, which is always present among the apoptotic cell population irrespective of the stimuli, shows the nuclear modification called "apoptosis/mitosis". Magnification: (a, c, f) 5500x; (b, d) 7000x; (e)12000x.
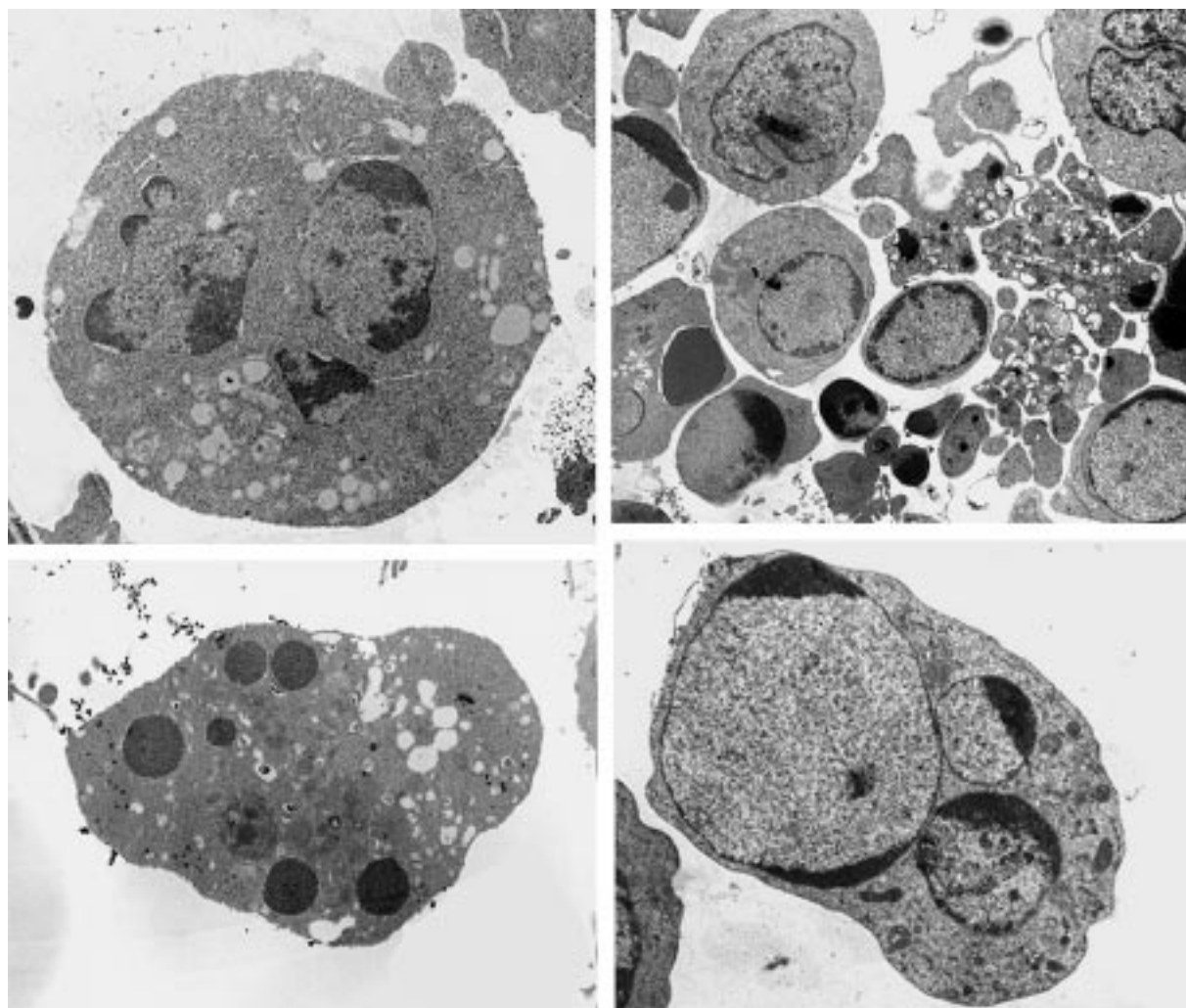
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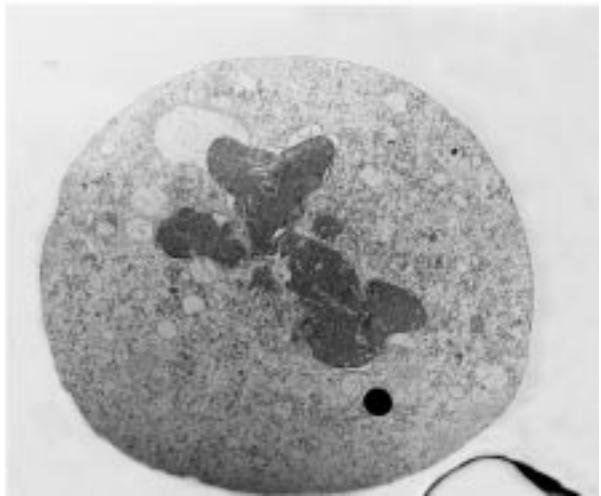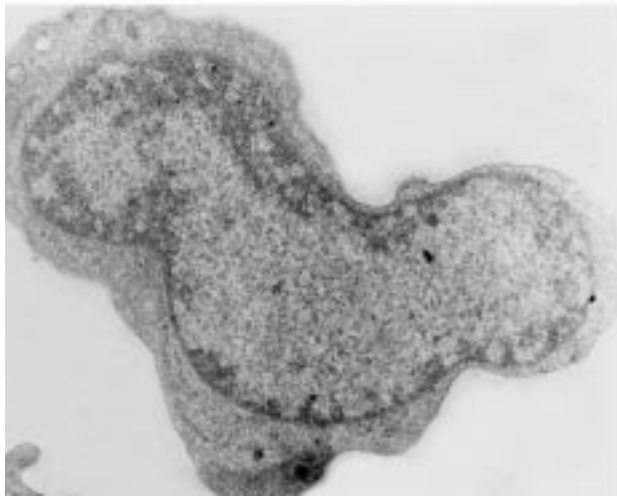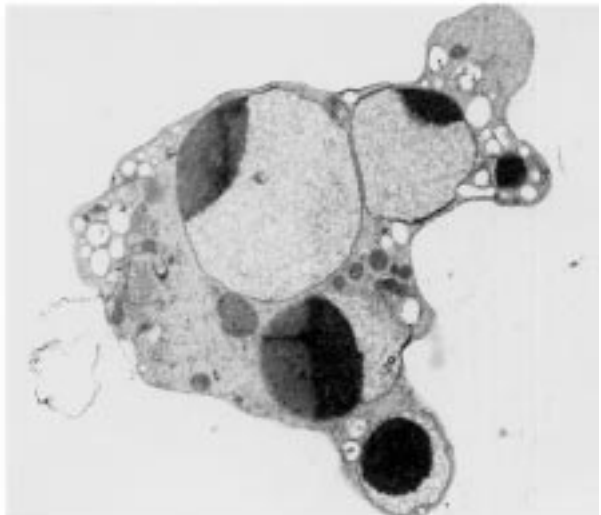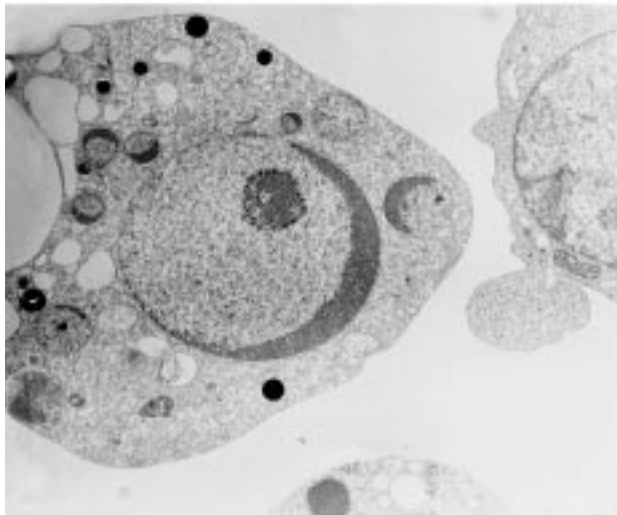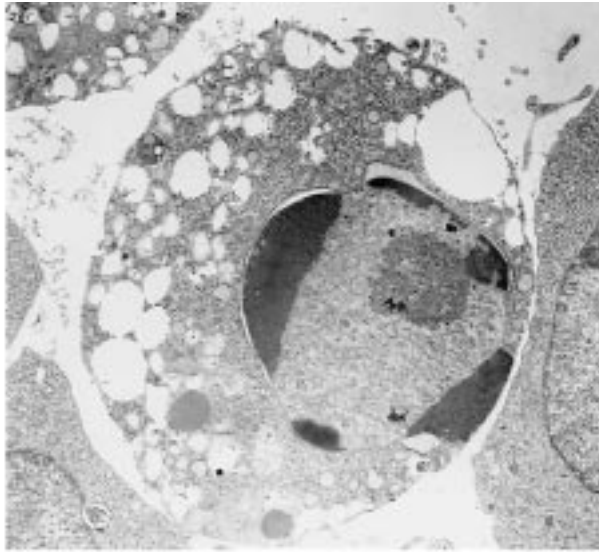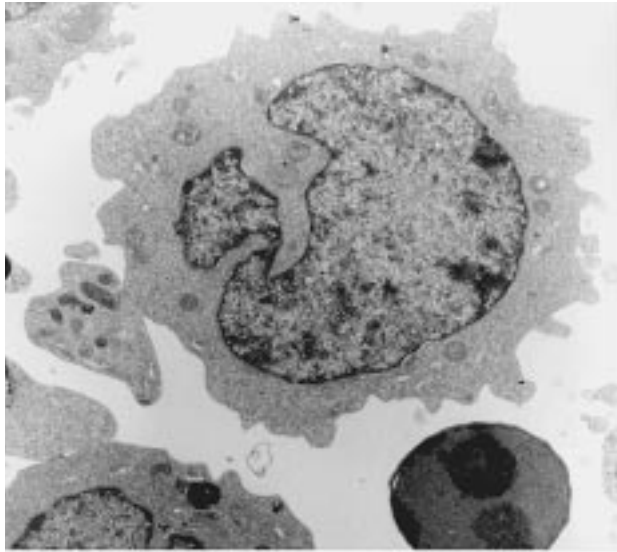
Fig. 3 - Transmission electron micrographs representative of apoptic U937 cells at early and late stages of the program following H$_2$O$_2$ (a,c) or puromycin (b,d) treatment. a) early apoptotic U937 cells showing chromatin condensation typical of budding fragmentation; b) total nuclear fragmentation in late apoptotic U937 cells: many nuclear fragments are spread in the cytoplasm; c) chromatin condensation and nuclear fragmentation by cleavage in early apoptotic U937 cells; d) nuclear fragments at the end of the fragmentation resulting from cleavage (late stage) are forming a cluster: some zip-like structures are visible at the point of contact (arrows). Percentages indicate the frequency that the type of nuclear fragmentation shown in each panel was observed in all experiments. Magnification: (a, c) 7000x; (b) 4200x; (d) 9000x.

quently observed in cells induced to apoptosis by puromycin. The percentage of each nuclear morphology also changed with time (Fig. 3 and Table I), thus suggesting that each picture is only an intermediate step during the programmed death. Therefore, our observations indicate that, within the same type of cell, morphological modifications of dying cells are related to the apoptogenic inducer and to the phase in the progression of apoptosis (Fig. 3 and Table I).

Cell shape modifications

During the apoptotic process, cell shape is dramatically altered; quite often many blebs protruding from the dying cells can be seen. As observed for the nuclear changes, the presence and extention of blebs are related to the inducer and to the phase in the progression of apoptosis. U937 cells treated with H$_2$O$_2$ progressively modify their shape (i.e. volume and roundness), but rarely are blebs formed; conversely, in puromycin-treated cells, blebs, very often deep in

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the cytoplasm, are highly frequent. Fig. 4 reports the time-course of blebbing appearance in puromycin treated U937 cells. As reported for the type of nuclear fragmentation, also for the presence and distribution of blebs, the apoptotic U937 population is not homogenous; in the same sample apoptotic cells without blebs, with blebs that squeeze the entire cell, superficial blebs all around the cell surface or blebs deeply imbedded in the cytoplasm but confined to one pole of the cell have been observed. The per-

centage of each type of blebbing is, once again, dependent on the inducer and on the phase of the apoptotic process.

At the ultrastructural level, a close relationship between the type of nuclear fragmentation and cell shape modifications or *vice versa*, can be found. A complete smooth surface of the apoptotic cells is linked to nuclear fragmentation by budding. With the same type of fragmentation, blebs can be also observed randomly or apically distributed on the
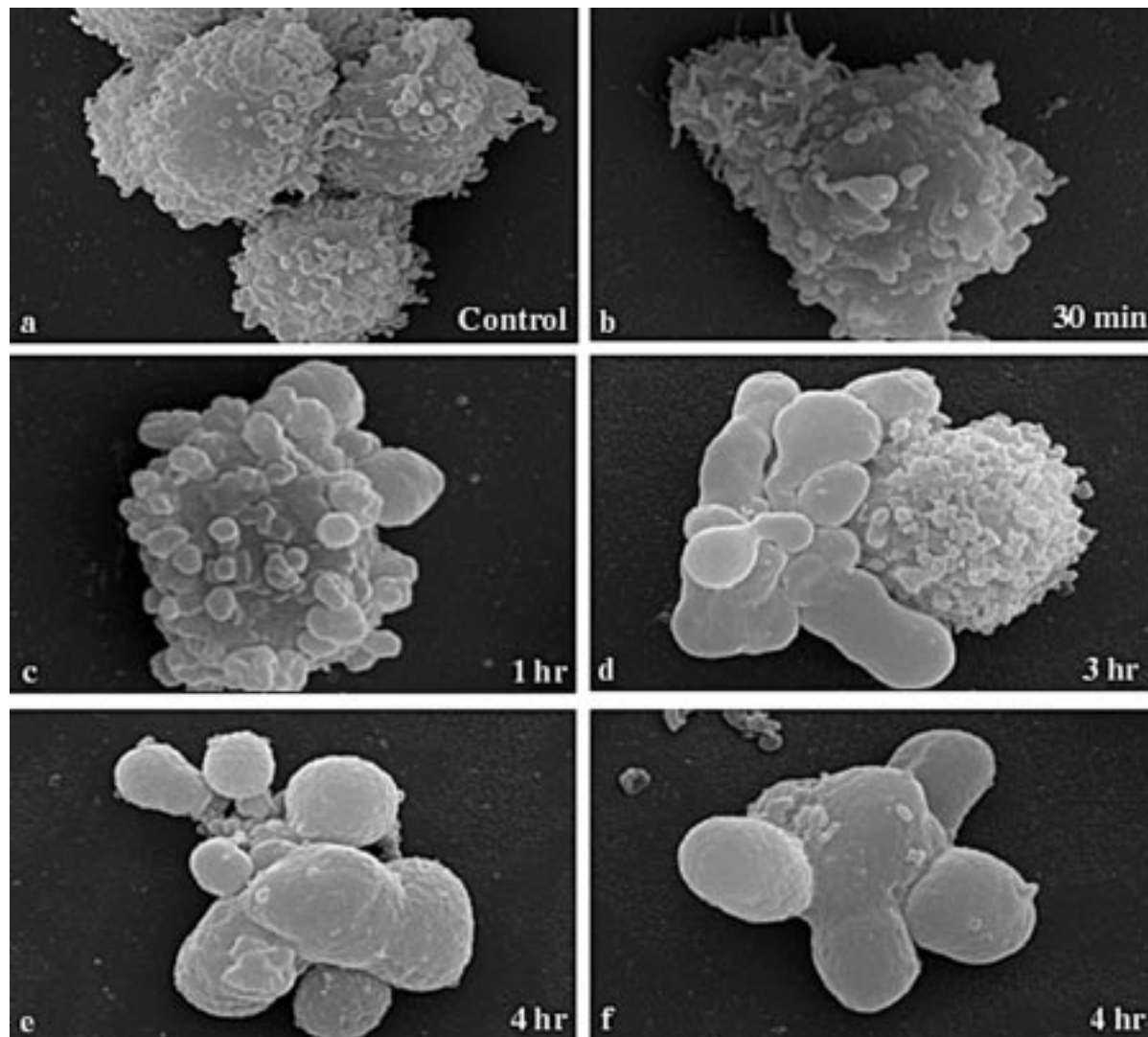

Fig. 4 - SEM micrographs showing the time course of blebbing in apoptotic U937 cells during 4hrs of continuous incubation with puromycin (10µg/ml). Normal U937 cells showing rough surfaces with small pseudopodia (a) that progressively round up starting at 1/2 hour of incubation with puromycin (b) and becoming more spherically at 1hr of incubation with puromycin (c). At the latest times of incubation (3 hrs) the cells are stretched and form big blebbing elongations (d) that after 4hrs of continuous incubation with puromycin have completely damaged the cell structure (e,f). Magnification: (a) 4200x; b) 8000x; (c) 8500x; (d,e) 5500x; f) 6000x

cell surface. On the other hand, apoptotic nuclear fragmentation by cleavage is always associated to *foamy* cell shape derived by the presence of deep blebs squeezing and destroying the entire cellular morphology (Fig. 5).

Cell surface modifications

We have examined the time course of modifications of PS and various carbohydrate residues (mannose, fucose, N-acetylglucosamine/galactose, N-acetylgalactosamine) on the plasma membrane of dying U937 cells after oxidative stress and inhibition of protein synthesis. Figures 6, 7, 8 and 9 show data from flow cytometry and from observation at conventional and confocal microscopy. The use of different techniques allowed us to obtain both qualitative and quantitative data.

Lectin binding sites on U937 cells (both normal and apoptotic) were only scarcely expressed with the exception of Con-A, WGA and PWM. The
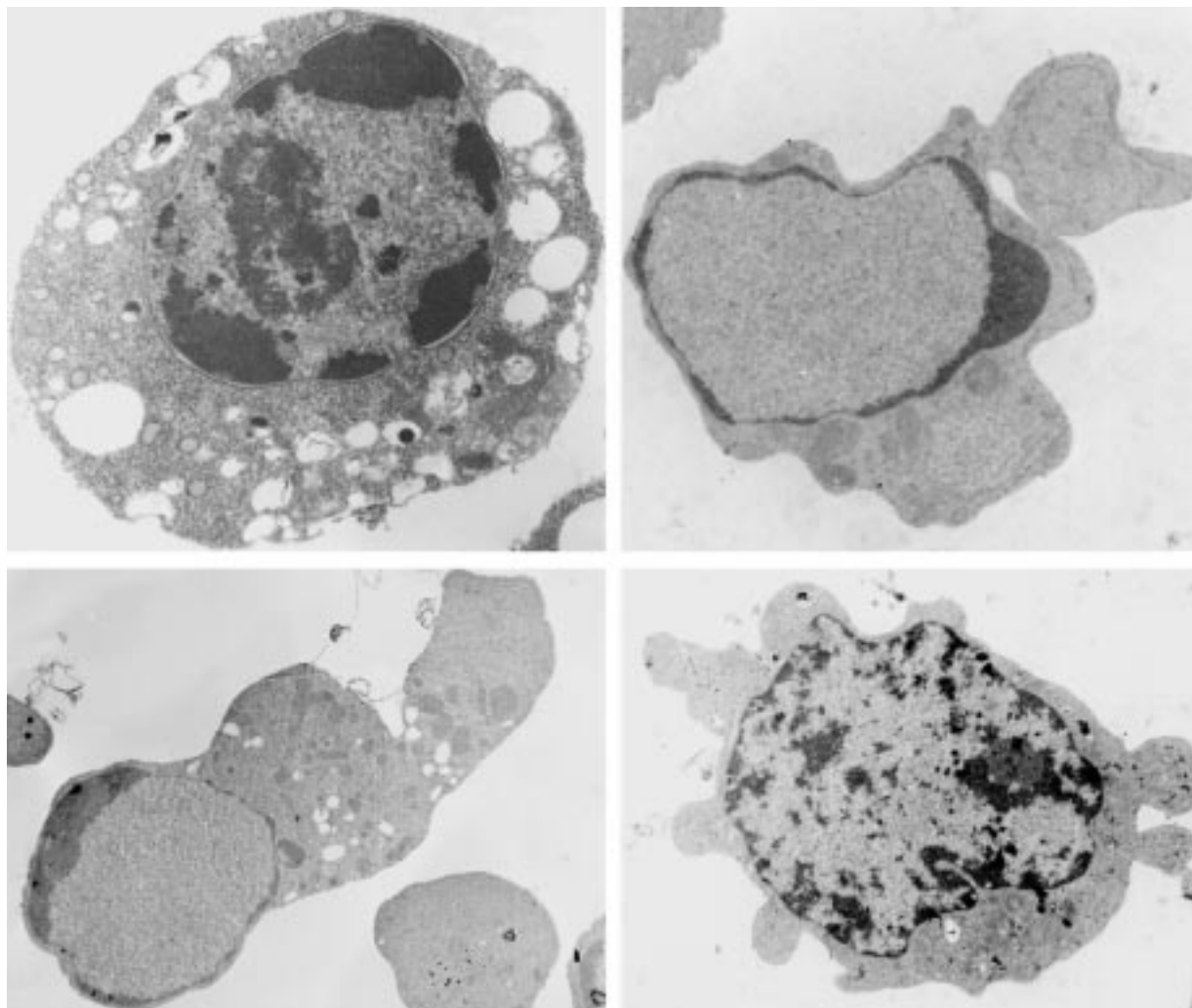

Fig. 5 - Transmission electron micrographs representative of blebbing apoptic U937 cells. The simultaneous observation of nuclear modifications and blebbing has been useful forcorrelating these two events: no blebs at all (a), apical blebbing, when the invaginations among blebs are deep in the cytoplasm but are only confined to one pole of the cell (d), and superficial blebbing, when the modifications occur in the entire cell but the constriction remains confined at the cell periphery (b) are all related to the nuclear fragmentation by budding. Total blebbing, when the entire cell is modified and squeezed in many bubbles (c) is observed with nuclear fragmentation by cleavage. Magnification: (a) 12000x; (b) 7000; (c) 5500; (d) 7000
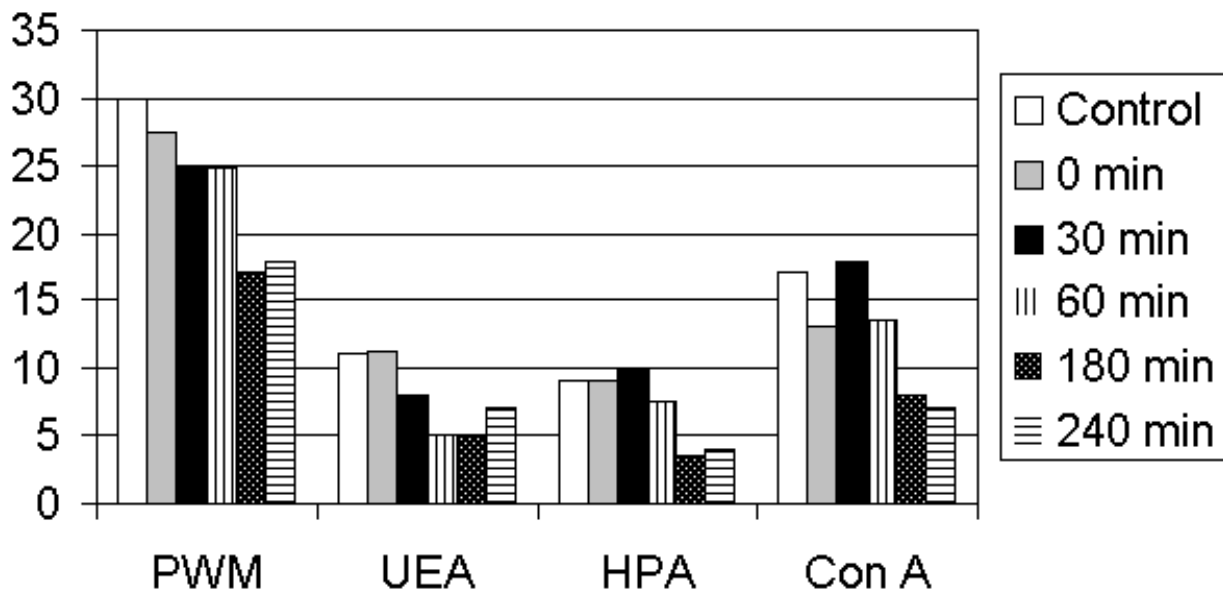
H2O2 (1 mM)
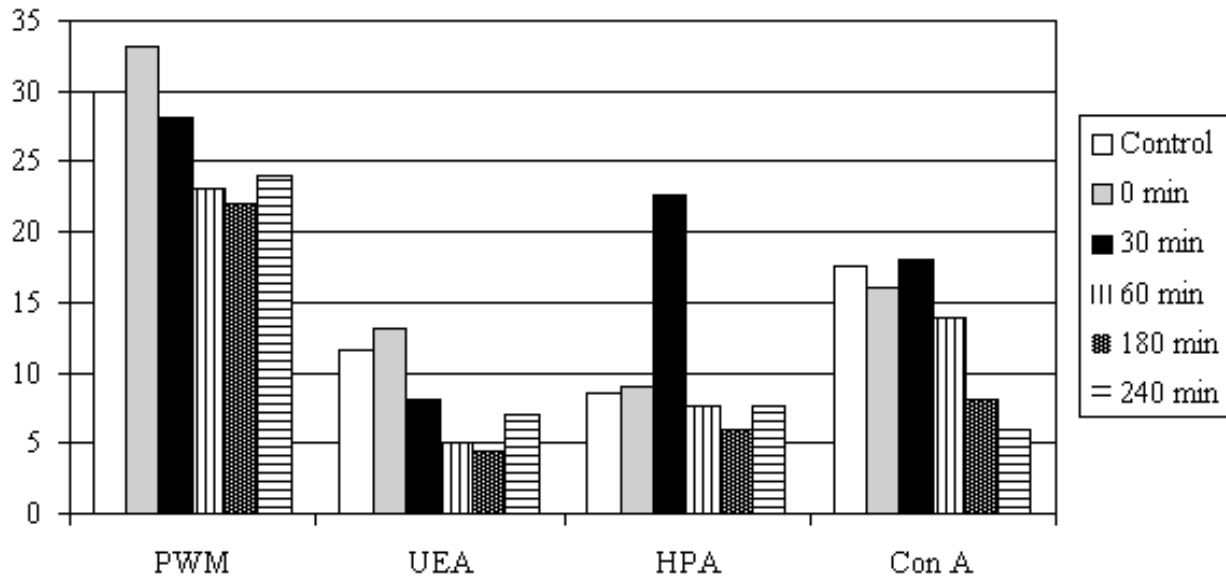
Puromicyn (10mg/ml)

Fig. 6 - Flow cytometry time course of the binding of lectin-FITC conjugate to the surface of normal and apoptotic U937 cells treated with H$_2$O$_2$ (1mM) (panel A) or puromycin (10μg/ml) (panel B). The lectins used were PWM *(Phytolacca americana),* UEA (*Ulex europaeus),* HPA *(Helix pomatia*), Con-A (Concanavalin-A). See Material and Methods for details. A progressive decrease in the exposure of sugar residues during the late stage of the apoptosis process was observed. Values are given as percentage of fluorescence. One experiment out of three performed is reported.
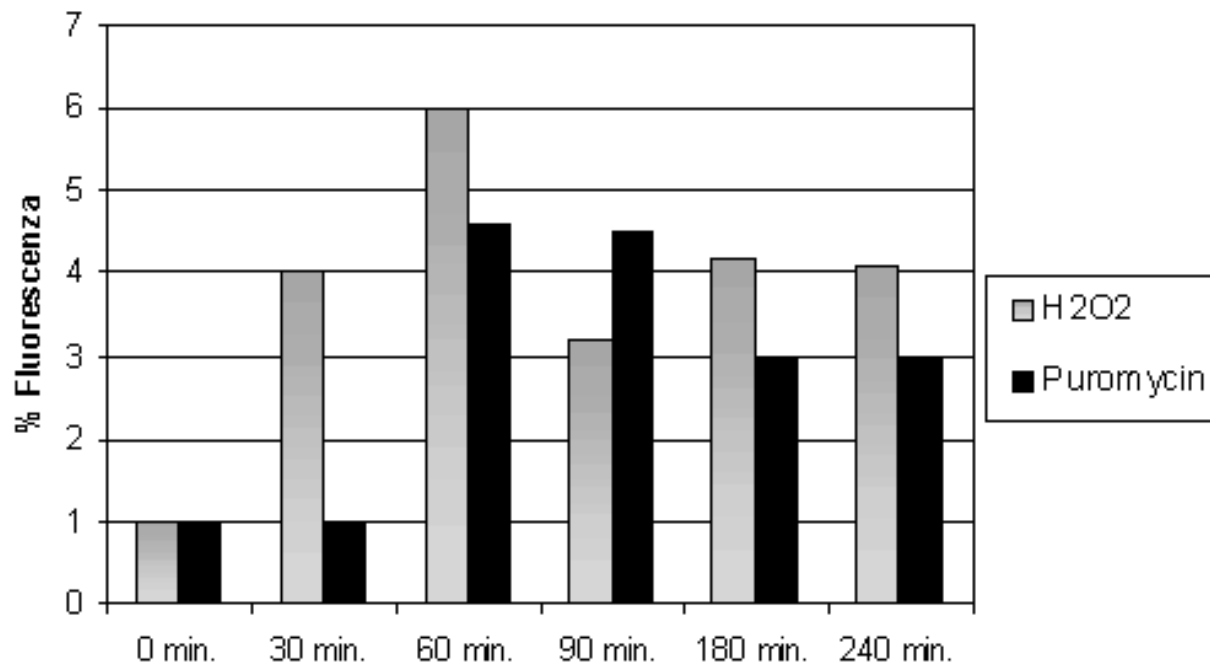
Fig. 7 - Flow cytometry time course of the binding of AnnexinV-FITC conjugate to the surface of normal and apoptotic U937 cells treated with H_2O_2 (1mM) or puromycin (10μg/ml). Following the apoptogenic treatment, PS exposure on the external leaflet of the plasma membrane increases during the early phase of the process and then the percentage of fluorescence decreases as the apoptotic program progresses. Values are given as percentage of fluorescence. One experiment out of three performed is reported.

amount and distribution of carbohydrate residues are dramatically modified during the apoptotic process in a time-dependent manner. However, the changes were independent of the inducer, and comparable cell surface modifications were found in both treatments. A common trend was found for all the lectins tested: their cell surface binding sites greatly decreased with time, thus indicating a loss of sugar residues. This decrement was particularly evident in the late phase of the apoptotic process. One exception was found for N-acetylgalactosamine residues, which increased dramatically, as detected by the increase of the fluorescent signal (about 3 times above the control values) during the early phase of the process. However, late apoptosis was characterized by a decrement of HPA binding sites in agreement with other lectin binding sites. The reason of this behaviour is, at present, still unknown. As far as the distribution of binding sites is concerned, the pattern of lectin bound to the plasma membrane of normal cells was (irrespective of lectins) uniformly distributed. A progressive change of lectin-FITC conjugates to

plasma membrane during apoptosis led to patch formations (Figs. 8, 9).

PS exposure was quickly expressed on the cell surface of U937 cells as randomly distributed fluorescence, in parallel with the appearance of apoptosis. Surprisingly, the amount of PS externalized on the cell surface was dependent on the inducer. In fact, cells that underwent oxidative stress externalized more PS on the outer leaflet of the plasma membrane than did the puromycin-treated U937 cells (Fig. 7).

By using double staining (P.I. and/or Hoechst for the nucleus and FITC-conjugated lectins or Annexin V for the surface modifications), nuclear and cell surface events were contemporaneously analyzed (Figs. 8, 9). Homogenously distributed fluorescence was present on the cell surface of normal cells, while a ligand distribution as patches was observed on the apoptotic cells. These modifications, which parallelled the nuclear changes, were gradually achieved during the apoptotic process. The early nuclear morphological modifications corresponded to a sligthly modified pattern distrib-
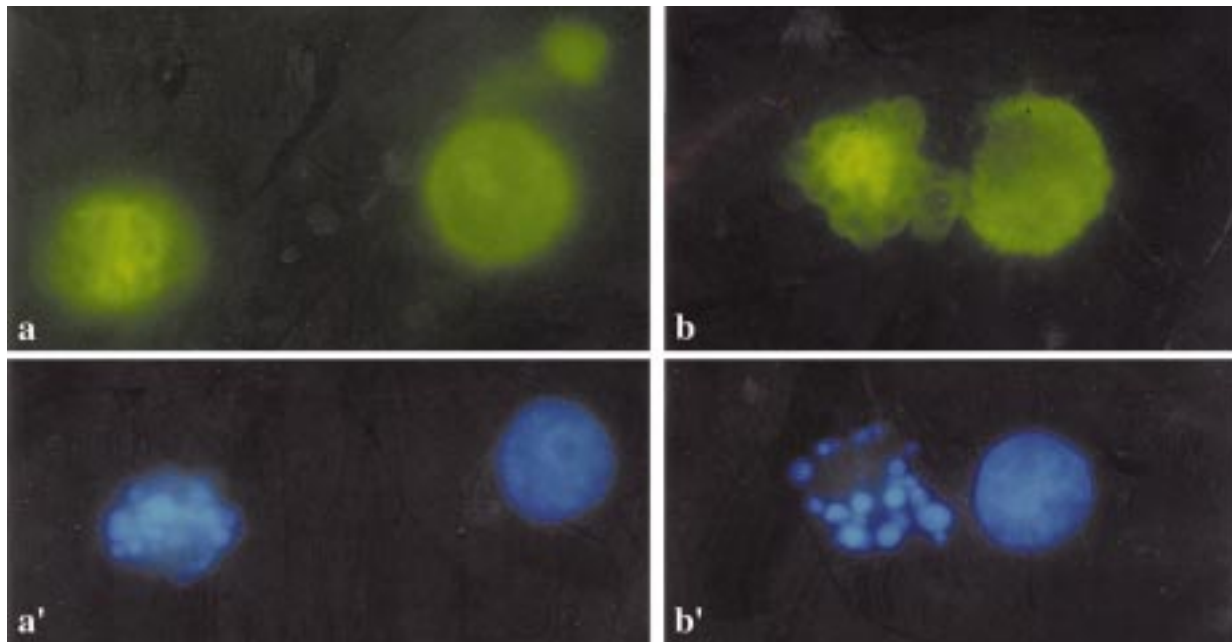
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Fig. 8 - Representative light micrographs of double labelling (WGA-Fitc and Hoechst) to U937 cells treated with H_2O_2 (1mM) (a,a') and puromycin (10µg/ml) (b,b'). The normal amount and distribution of carbohydrate residues modifies (independent of the inducer) during the apoptotic process in a time-dependent manner with comparable cell surface modifications for both treatments. Sugar residues are not concentrated on cell surface areas corresponding to nuclear fragments of highly condensed chromatin. Magnification: 1000x.
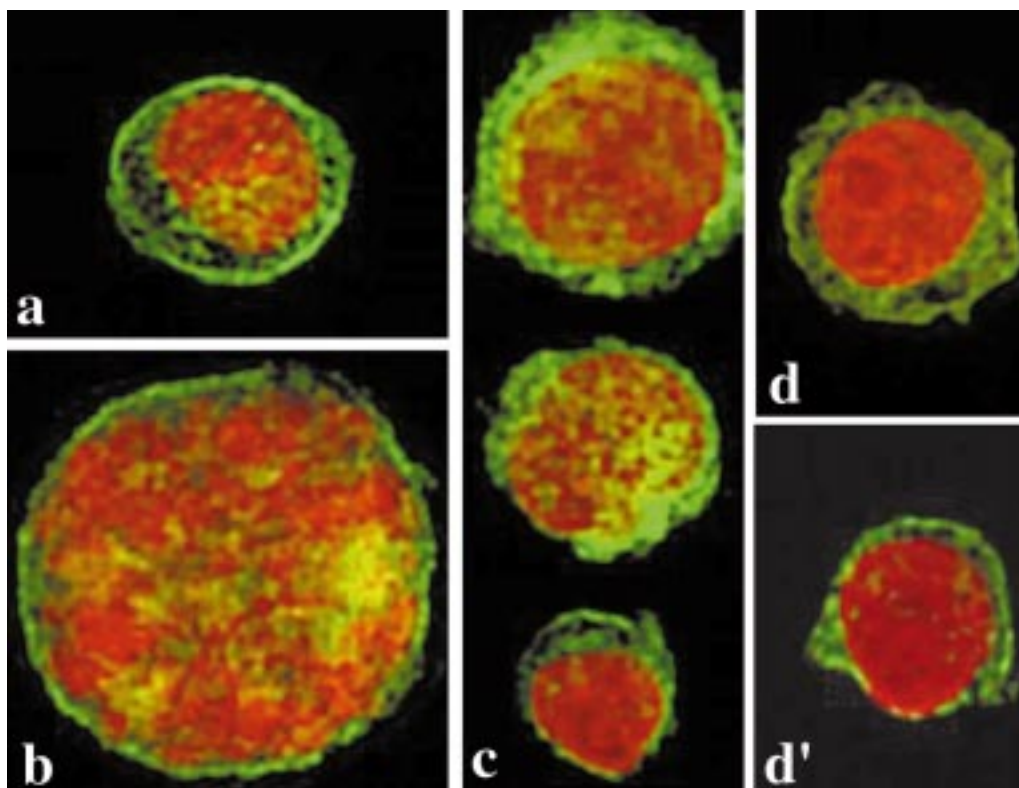
Fig. 9 - Representative confocal micrographs of double labelling (WGA-FITC and Iodure propide) to U937 cells treated with puromycin (10µg/ml) (c) and H_2O_2 (1mM) (d,d'). Two aspects are predominant: i) the modifications (quantity and distribution) of the sugar residues on the cell surface of normal cells (a) and on the apoptotic ones; ii) the similarity between the patches distribution of the fluorescence of the late apoptotic cells and the mitotic ones (b). Magnification: 2000x.

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ution of fluorescent conjugates. In particular, during the "early" apoptotic phase, fluorescence corresponded to nuclear areas with not condensed chromatin. At the late apoptotic phase, when chromatin was condensed, the intensity of fluorescence decreased and the pattern changed to patches. Surprisingly, a similar pattern was observed in mitotic cells (Fig. 9). It is worthwhile to mention the differences among mature apoptotic U937 cells following oxidative stress and those treated by puromycin. The latter, in fact, showed lower fluorescence for both lectin and Annexin-V conjugates. This progressive reduction of fluorescence was evident in particular in the blebs, very common in the puromycin treated cells.

DISCUSSION

In this study, the time-course of morphological modifications of U937 cells induced to apoptosis by two treatments with different cellular targets, i.e. puromycin or H_2O_2 has been described. The research was focused on the relation between the onset of nuclear modifications and the onset of cell surface changes, both sites of major morphological changes during the apoptotic process.

Virtually every cell constitutively expresses a core program controlling the proper execution phases of the apoptotic program. Cell death is more often defined by the use of morphological criteria (Leist and Nicotera 1997; Searle *et al.* 1982). For example, chromatin condensation has been for a long time considered to be a requisite for the detection of apoptosis (Gavrieli *et al*. 1992; Falcieri *et al.* 1994b; Leist and Nicotera 1997). However, all the morphological changes so far reported in the literature (Allen 1987; Falcieri *et al.* 1994a; Madeo *et al.* 1997; Di Baldassarre *et al.* 2000) do not describe the morphology of apoptotic phenomena. In fact, the known morphology of apoptosis give us only an individual framework? corresponding to biochemical events during the execution of the apoptotic program. This is the reason why many different morphologies of apoptosis are described (Dini *et al.* 1996). On the other hand, the concept of a programmed death is not always linked to a specific morphology. For example, very often, in non-vertebrate systems, programmed cell death does not display an apoptotic-like morphology (Leist and Nicotera, 1997; Schwartz and Osborne, 1993).

However, opposite results are shown in a recent paper by Cikala *et al.* (1999). They report that in hydra, cell death is morphologically indistinguishable from apoptosis in higher animals. In addition, hydra polyps express two genes with strong homology to members of the caspase-3 family, showing caspase-3-specific enzyme activity (Cikala *et al.* 1999). It has been recently hypothesized that different types of cell death share common mechanisms in the early phase, whereas activation of caspases determines the phenotype of cell death supporting the correlation between biochemical events and cell morphology (Saraste and Pulkki, 2000). Moreover, in a yeast mutant, a number of morphological and molecular indicators of apoptosis that are typical of the metazoan cells, are present: i.e. exposure of PS on the outer leaflet of the cytoplasmic membrane, DNA breakage, chromatin condensation and fragmentation, and even a series of tiny buds that can be considered the equivalent of apoptotic bodies (Madeo *et al.* 1997).

We have described here in the U937 cell lines that each different morphology is related to the apoptogenic stimulus and to the step in the execution of the apoptotic process. It derives from this that progressive modifications of the nucleus can be used to distinguish among early, mature or late apoptotic cells. However, these criteria are not feasible for apoptotic bodies, beeing very heterogenous structures due to the modality of their formation (by cytoplasmic fragmentation). The morphology of apoptotic bodies is not representative of the entire apoptotic cell, because, during cell fragmentation, various components of the cytoplasm are segregated in different places (Bonanno *et al.* 2000). Very often, the cytoplasm in the blebs does not contain organelles or remnants of organelle-like vescicles, etc.

The apoptogenic inducer affects cell shape (blebbing), exposure and cell surface distribution of PS and sugar residues. However, modifications at the level of the plasma membrane seem to be a compulsory event of the apoptotic program for this cell line. In fact, apoptotic U937 cells, despite their cell surface changes, are never phagocytosed in *in vitro* or *in vivo* tests (Dini 2000). In spite of the fact that PS and sugar residues exposure are responsible for the proper recognition of apoptotic cells, we found that their amount on the cell surface decreased at the later stages of apoptosis. Our data (at least for PS externalization) are in agreement with the work of Spano *et al.* (2000) who

reported a similar decrement of PS exposure. PS has been reported to be a transient event with an expression peak during the early stages of apoptosis. Moreover, modifications of the sugar residues expression appear to be a more complex phenomenon including increase and/or decrease of different residues at the different steps of programmed death. A hierarchy of progressive exposure of sugar residues during apoptosis has not yet been reported (Russell *et al.* 1998). In addition, from the localization studies, plasma membrane delimiting detaching blebs always appears to be different (decreasing amount and distribution of both PS and sugar residues) from the rest of the cell (Bonanno *et al.* 2000). Only a partial explanation could be found in the release of membrane during apoptosis (Zhang *et al.* 1998).

As the apoptotic phenomenon of U937 cells progresses over the early stages, it causes extensive formation of blebs that, concomitantly, loose some typical markers of apoptosis, such as PS or sugar residues. One major candidate to link nuclear modifications, blebbing formation and cell surface changes might be the cytoskeleton. Changes in the cytoskeletal organization have been extensively described to occur during apoptosis in different biological situations (Wyllie et al 1980; Ijiri and Potten 1987), and it is now clear that the reorganization of microtubules is an integral part of the apoptotic process (Pittman *et al.* 1994; Ireland and Pittman 1995). Moreover, in apoptotic cells, cytokeratin filaments aggregate in the early stage of the process, while during later stages they are degraded (Tinnemans *et al.* 1995). It was proposed that, membrane blebbing is a discrete subprogram operating during mammalian apoptosis which can lead to cell death via caspase-independent mechanisms (Mc Carthy *et al.* 1997), although surface blebbing is not essential for apoptosis completion and can be dissociated from it (Huot *et al.* 1998). Membrane blebbing is thus a primary modification of cell toxicity which is not necessarily linked to apoptosis.

It may be also kept in mind that evidence has been presented implying a role of conventional and unconventional myosins in the formation of blebs in serum-deprived cells (Mills *et al.* 1998); and of a relationship between membrane lipid asymmetry and cytoskeletal alterations, and between actin microfilaments and dynamics of the plasma membrane and cytoplasm modifications related to the formation of blebs (Kulkarni *et al.* 1994).

Finally, it is worth noting that physiological and damaging events have recently been reported to activate two different pathways of apoptotic signalling. The two pathways converge at the activation of caspase-3, the key effector of the execution phase of apoptosis, thus giving rise to apoptosis (Ghibelli and Coppola, 2000). The knowledge that different biochemical routes exist could explain the reason for the different morphological appearances. With regards to this latter aspect, it should be kept in mind that the inducers used in this study interfere with different cellular targets: the first inducer (H202) causes an imbalance in the redox condition of the cell, thus triggering apoptosis by an oxidative stress, while the second induces apoptosis via a non-oxidative pathway.

Summing up, our findings demonstrate that in the U937 cell lines cellular modifications (i.e. percentage of apoptosis, chromatin condensation, blebbing) depend on the inducing treatment. In fact, although the final stages of apoptosis are morphologically indistinguishable, the morphology of early and mature phases of apoptosis is strictly determined by the inducer. Oxidative stress induces nuclear budding and occasional membrane blebs, whereas puromycin cause nuclear cleavage and a large amounts of blebs. On the other hand, in the late phase of the apoptotic process, we observed a complete nuclear and/or cytoplasmic fragmentation irrespective of the apoptotic treatment (see Table I). Quantitative modifications of PS and sugar residues are not related to the apoptotic treatment although topographic variations have been observed. It is interesting to note that blebs formation and distribution are closely related to the modality of nuclear fragmentation since they are significantly present in the cleavage modality (see Table I).

In conclusion these morphological data should improve the detection of apoptotic cells *in vivo* (i.e. in tissues, organs), in particular during the early stages of the process, leading to a more precise quantification of apoptosis. A precise apoptotic recognition is a crucial point in the evaluation of efficiency of antiproliferative drugs such as antiblastic or immunosuppressive molecules.

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REFERENCES

Allen, T.D.: Ultrastructural aspects of cell death. In Perspective on mammalian cell death. *C.S. Potten editor.* Oxford university Press 39-65, 1987.

Bonanno, E., Ruzittu, M., Carlà, E.C., Montinari, M.R., Pagliara, P., and Dini, L.: Cell shape and organelle modification in apoptotic U937 cells. Eur .J. Histochem. *44*, 237-246, 2000.

Cikala, M., Wilm, B., Hobmayer, E., Boettger, A., and David, C.: Identification of caspases and apoptosis in the simple metazoan Hydra. Current Biology *9*, 959-962, 1999.

Di Baldassarre, A., Secchiero, P., Grilli, A., Celeghini, C., Falcieri, E., and Zauli, G.: Morphological features of apoptosis in hematopoietic cells belonging to the T-lympocyte lineages. Cell. Mol. Biol. (Noisy-le-grand) *46*, 153-161, 2000.

Dini, L.: Recognizing death: liver phagocytosis of apoptotic cells. Eur. J. Histochem. *44*, 217-227,2000.

Dini, L., Autuori, F., Lentini, A., Oliverio, S., and Piacentini, M.: The clearance of apoptotic cells in the liver is mediated by the asialoglycoprotein receptor. FEBS *296*, 174-178, 1992.

Dini, L., Coppola, S., Ruzittu, M.T., and Ghibelli, L.: Multiple pathways for apoptotic nuclear fragmentation. Exp. Cell Res. *223*, 340-347, 1996.

Fadok, V.A., and Henson, P.M. (1998). Apoptosis: getting rid of the bodies. Current biology *8*, R693-R695.

Falcieri, E., Gobbi, P., Zamai, L., and Vitale, M.: Ultrastructural features of apoptosis. Scanning Microsc. *8*, 653-655, 1994a.

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

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S.A.: Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J. Cell Biol. *119(3),* 493-501, 1992.

Ghibelli, L., and Coppola S.: GSH extrusion and the mitochondrial pathway of apoptotic signalling. Biochem. Soc. Trans. *28,* 56-61, 2000.

Huot, J., Houle, F., Rousseau, S., Deschesnes, R.G., Shah, G.M., and Landry, J.: SAPK2/p38-dependent F-actin reorganization regulates early membrane blebbing during stress-induced apoptosis. J. Cell Biol. *143,* 1361-1373, 1998.

Ijiri, K., and Potten, C.S.: Further studies on the response of intestinal crypt cells of different hierarchical status to eighteen different cytotoxic agents. Br. J. Cancer *55,* 113-123, 1987.

Ireland, C.M., and Pittman, S.M.: Tubulin alterations in taxol-induced apoptosis parallel those observed with other drugs. Biochem. Pharmacol. *49,* 1491-1499, 1995.

Kulkarni, G.V,. and McCulloch, C.A.G.: Serum deprivation induces apoptotic cell death in a subset of Balb/c 3T3 fibroblasts. J.Cell Sci. *107,* 1169-1179, 1994.

Kumar, S., and Harvey, N.L.: Role of multiple cellular proteases in the execution of programmed cell death. FEBS Lett. *375,* 169-73, 1995.

Leist, M., and Nicotera, P.: Breakthroughs and views. The shape of cell death. Biochim. Biophys. Res. Commun. *236,* 1-9, 1997.

Madeo, F., Frohlich, E., and Frohlich, K.: A yeast mutant showing diagnostic markers? of early and late apoptosis. J.Cell Biol. *139,* 729-734, 1997.

McCarthy, N.J., Whyte, M.K.B., Gilbert, C.S., and Evan, G.I.: Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. J. Cell Biol. *136,* 215-227, 1997.

Mills, J.C., Stone N.L., Erhardt J., and Pittman SM. Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. J.Cell Biol. *140,* 627-636, 1998.

Morris R.G., Hargreaves A.D., Duvall E., and Wyllie A.H.: Hormone induced cell death. Surface changes in thymocytes undergoing apoptosis. Am. J. Pathol. *115,* 426-436, 1984.

Nagata, S.: Apoptotic DNA fragmentation. Exp. Cell Res. *256,* 12-18, 2000.

Pittman, S.M., Strickland D., and Ireland C.M.: Polymerization of tubulin in apoptotic cells is not cell cycle dependent. Exp. Cell Res. *215,* 263-272, 1994.

Platt, N., Pedro da Silva, R., and Gordon, S.: Recognizing death: the phagocytosis of apoptotic cells. Trends in Cell Biology *8,* 365-372, 1998.

Rudel, T., and Bocock, G.M.: Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. Science *276*, 1571-1574, 1997.

Russell, L., Waring, P., and Beaver, J.P. Increased cell surface exposure of fucose residues is a late event in apoptosis. Biochem. Biophys. Res. Commun. *250,* 449-453, 1998.

Saraste, A., and Pulkki, K.: Morphologic and biochemical hallmarks of apoptosis. Cardiovasc. Res. *45,* 528-537, 2000.

Savill, J.: Phagocytotic docking without shocking. Nature *392,* 442-443, 1998.

Schwartz, L.M., and Osborne, B.A.: Programmed cell death, apoptosis and killer genes. Immunol. Today *14,* 582-590, 1993.

Searle, J., Kerr, J.F.R., and Bishop, C.J.: Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathol. Annu. *17,* 229-259, 1982.

Spano, A., Sciola, L., Monaco, G., and Barni, S. Relationship between actin microfilaments and plasma membrane changes during apoptosis of neoplastic cell lines in different culture conditions. Eur. J. Histochem. *44,* 225-267, 2000.

Tinnemans, M.M.F.J., Lenders, M.H.J.H., ten Velde, G.P.M., Ramaekers, F.C.S., and Schutte, B. Alterations in cytoskeletal and nuclear matrix-associated proteins during apoptosis. Eur. J. Cell Biol. *68,* 35-46, 1995.

Wyllie A.H., Kerr J.F.K., and Currie A.R.: Cell death: the significance of apoptosis. Int. Rev. Cytol. *68*, 251-306, 1980.

Zhang, J., Driscoll, T.A., Hannun, Y., and Obeid, L.: Regulation of membrane release in apoptosis. Biochem. J. *334,* 479-485, 1998.