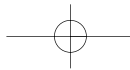

ORIGINAL PAPER

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Rearrangement of nuclear ribonucleoproteins and extrusion of nucleolus-like bodies during apoptosis induced by hypertonic stress

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SUMMARY

Short-term hypertonic (HT) stress induces apoptotic cell death in human EUE cells in culture, as observed by electron microscopy, agarose-gel electrophoresis of low-molecular-weight DNA, DNA flow cytometry and annexin-V-propidium iodide double-staining. During HT-induced apoptosis, nuclear ribonucleoprotein (RNP)-containing structures undergo rearrangement, with the formation of Heterogeneous Ectopic RNP-Derived Structures (HERDS) which pass into the cytoplasm, as already reported for other examples of spontaneous and drug-induced apoptosis. Of special interest was the observation that nucleolus-like bodies (NLBs) which resemble morphologically nuclear functional nucleoli may be extruded into the cytoplasm of apoptotic cells and are observed inside the cytoplasmic fragments blebbing-out at the cell surface; these NLBs still contain immunodetectable nucleolar proteins (such as fibrillarin). This is an additional example of RNP-containing structures of nuclear origin which are extruded from the nucleus, in an almost "native" form, during apoptosis.

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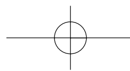
INTRODUCTION

Eukaryotic cells are able to react to a wide spectrum of environmental stresses through changes in gene expression and the synthesis of the so-called stress proteins (Morimoto *et al.*, 1994). These stress-induced functional alterations may be paralleled by changes in the structural and kinetic features of cells, like those observed in human embryonic epithelial (EUE) cells as a consequence of the hypertonic (HT) stress (Pellicciari *et al.*, 1989, 1991, 1995). In fact, upon long-lasting (at least a few days) exposure to a HT culture medium, EUE cells increase their size and protein content, modify their gene expression with an increased synthesis of a 33 kDa stress protein (which proved to be aldose reductase: Giuliani *et al.*, 1991; Ferraretto *et al.*, 1993; Pellicciari *et al.*, 1993), and decrease their proliferation potential with the exit of most of the cells from the cycle into a G0 quiescent state (Pellicciari *et al.*, 1989, 1991). At least some of these features relate to the resistance against the HT stress: namely, the increased expression of aldose reductase may directly influence the synthesis of organic osmolytes necessary for resisting



the high osmotic pressure (Ferraretto *et al.*, 1993), while the cells arrested in G0 are known to be less prone to environmental damage (Pellicciari *et al.* 1991, 1995).

However, at the beginning (within 6 h) of the exposure to the HT medium, about 50% of EUE cells could not resist this stress and died (Pellicciari *et al.*, 1989). We therefore decided to investigate the mode of HT-induced cell death, namely whether hypertonicity may induce apoptosis (as reported in the literature for other cell types: Parolari *et al.*, 1997; Hizoh *et al.*, 1998) and whether EUE die in a given phase of the cell cycle.

Since hyperosmolarity was reported to affect transcription and protein synthesis (Petronini *et al.*, 1986, 1987; Pellicciari *et al.*, 1993), we paid special attention to the restructuring of the nucleolus and of other nuclear ribonucleoprotein (RNP) containing structures (i.e. perichromatin fibrils PF, perichromatin granules PG, and interchromatin granules IG) which are involved in the synthesis and processing of mRNA (Fakan, 1994; Puvion and Puvion-Dutilleul, 1996): actually, severe structural rearrangement of nuclear RNPs occurs during apoptosis (Biggiogera *et al.*, 1997a, b; 1998) or in relation to altered transcription (Biggiogera and Pellicciari, 2000).

MATERIALS AND METHODS

Cells and culture conditions

EUE cells from normal human embryonic epithelium (Terni and Lo Monaco, 1958) were grown either in 25 cm^2 plastic flasks or on glass coverslips in multi-well dishes in D-MEM medium containing 10% fetal bovine serum, 2 mM glutamine and 100 units/ml of streptomycin and penicillin (all the reagents and disposable material were from Celbio S.r.l., Milan, Italy).

Cells were allowed to grow for at least 24 h in an isotonic complete medium, which was then replaced with an HT medium for 6 h. The HT medium was obtained by increasing the NaCl concentration (from 0.137 M to 0.246 M) in the saline solutions used as a component of the culture medium whose final osmolarity was 495 mOsm, instead of the isotonic 305 mOsm (measurements were taken by an osmometer: Knauer, Berlin, Germany).

Electron microscopy

EUE cells grown in flasks were detached by trypsin-EDTA (0.5% trypsin, 0.05% EDTA in PBS) and fixed either in 2% glutaraldehyde (1 h, 4°C) or in 4% paraformaldehyde (2 h, 4°C) in Sörensen buffer. Glutaraldehyde-fixed specimens were post-fixed with 1% OsO_4 for 1 h, 4°C. Paraformaldehyde-fixed specimens were treated for 15 min with 0.5M NH_4Cl in PBS at 4°C to block free aldehyde groups. All the samples were then dehydrated and embedded in LR White resin.

For immunoelectron microscopy, ultrathin sections on formvar-carbon coated nickel grids were incubated for 3 min on a drop of normal goat serum (NGS) diluted 1:100 in PBS and treated according to one of the following procedures:

- The primary anti-SC-35 (Fu and Maniatis, 1992) monoclonal antibody (purchased from Sigma Chem. Co., St. Louis MO, USA) was diluted 1:25 in PBS containing 0.1% bovine serum albumin (BSA) and 0.05% Tween 20 and the incubation took place overnight at 4°C. After rinsing with PBS-Tween and PBS, the grids were incubated with NGS as above. The secondary antibody, a goat anti-mouse IgG coupled with 20 nm colloidal gold (MultiLab, Fetcham, UK), was diluted 1:10 in PBS, and the incubation was carried out for 30 min at room temperature.
- The primary anti-Sm autoimmune antibody (a kind gift of Dr. Milvia Lotzniker, IRCCS San Matteo, Pavia, Italy) recognizing two bands of 34-36 kDa (see Biggiogera *et al.*, 1999) was diluted 1:200 in PBS containing 0.1% BSA and 0.05% Tween 20 and salmon sperm DNA to inhibit the anti-DNA activity of such sera. As a secondary antibody, a goat anti-human IgG coupled with 20 nm colloidal gold (Aurion, Wageningen, The Netherlands), was used diluted 1:10 in PBS for 30 min at room temperature.
- The primary anti-hnRNP antibody raised in chicken (Martin and Okamura, 1981) was diluted 1:3000 in PBS containing 0.1% bovine serum albumin (BSA) and 0.05% Tween 20 and the incubation took place for overnight at 4° C. After rinsing for 3 min with PBS-Tween-BSA, the grids were incubated with a secondary anti-chicken antibody raised in rabbit (1:100) for 30 min at room temperature, rinsed in PBS for 5 min and incubated with NGS as above. As the tertiary antibody, we used a goat anti-rabbit antibody coupled

with 20 nm colloidal gold (MultiLab, Fetcham, UK) which was diluted 1:10 in PBS; the grids were incubated for 30 min at room temperature.

As a control, some sections were incubated in the absence of the primary antibodies and then treated with colloidal gold-labeled secondary antibodies. The grids from glutaraldehyde-osmium fixed specimens were routinely stained with uranyl and lead, while the immunolabeled sections were stained with the EDTA regressive technique (Bernhard, 1969).

Stained specimens were observed with a Zeiss EM900 electron microscope equipped with a 30µm objective aperture and operating at 80kV.

Fluorescence immunocytochemistry and confocal laser scanning microscopy

Cells grown on glass coverslips were fixed with 70% ethanol at 4°C for 30 min, then rehydrated with PBS, and permeabilized with 1% bovine serum albumin (BSA) and 0.5% Tween 20 in PBS for 10 min. To label the nucleolar protein fibrillarin, we used an autoimmune serum (kind gift of Dr. Milvia Lotzniker) diluted 1:800 with the BSA-Tween solution; after 1 h incubation, cells were washed with PBS and incubated for 45 min with a FITC-labeled anti-human IgG (Dako, Glostrup, Danemark). Cells were finally counterstained for DNA with 0.1 µg/ml DAPI (Sigma Chem. Co) in PBS for 15 min and mounted in glycerol:PBS (1:1).

For fluorescence microscopy, an Olympus BX50 microscope equipped with an 100 W mercury lamp was used, under the following conditions: 330-385 nm excitation filter (excf), 400 nm dichroic mirror (dm) and 420 nm barrier filter (bf), for DAPI; and 450-480 nm excf, 500 nm dm, and 515 nm bf for FITC. Photomicrographs were taken using Scotch 320T films.

For confocal laser scanning microscopy, we used a Leica TCS-SP system mounted on a Leica DMIRBE inverted microscope and equipped with an argon laser excitation. 0.5 µm spaced optical sections were recorded using a 63x oil immersion objective.

Agarose gel electrophoresis of low molecular weight DNA

To assess DNA fragmentation, cell pellets were lysed in 10 mM Tris-HCl buffer, pH 8, 10 mM EDTA, 0.5% Triton-X 100. Lysates were cleared by centrifugation at 13,000 g for 10 min, and proteinase K was added to a final concentration of 120 µg/ml. Following an overnight incubation at 50°C, cell lysates were extracted with phenol and then with chlorophorm:isoamyl alcohol (24:1). DNA was precipitated by addition of 2.5 volumes of ethanol, and resuspended in 10 mM Tris-HCl buffer, pH 8, 1 mM EDTA, containing 100 Kunitz units/ml RNase A, prior to electrophoresis on 1.2% agarose containing 0.5 µg/ml ethidium bromide. The DNA extracted from $2.5x10^6$ cells was loaded in each well.

Flow cytometry

Control and HT-treated EUE cells were detached by trypsin-EDTA (as reported above). Aliquots of unfixed cells were either stained for DNA (30 min with 50 µg/ml PI in water containing 0.001% Nonidet NP40 and 100 units/ml RNase type A; all the reagents from Sigma Chemical Co., St. Louis, MO) or resuspended for 10 min in complete medium containing FITC-conjugated Annexin V (3 $µl/10^6$ cells, Bender MedSystem, Prodotti Gianni, Milan, Italy) and 2 µg/ml PI (for details, see Pellicciari *et al.*, 1997).

Measurements were taken with a FACStar flow cytometer (Becton Dickinson, San José, CA, USA), equipped with an argon laser excitation (power 200 mW) at 488 nm, a 510-540 nm interference filter for the detection of FITC green fluorescence and 610 nm longpass filter for the PI red fluorescence detection. In each experiment, at least 20,000 events were measured in the gated regions used for calculations.

RESULTS

After 6 h exposure to the HT medium, apoptosis was found. Cytometric measurements after double staining with FITC-labeled Annexin V and PI (Fig. 1a,a') showed that more than 30% of EUE cells have membrane apoptotic characteristics; in addition, in DNA histograms (Fig. 1b,b'), a distinct sub-G1 peak was observed suggesting that most of the cells die in the G0/1 range of DNA content. The internucleosomal DNA degradation was confirmed by the typical laddering after agarose gel electrophoresis (Fig. 1c).

By electron microscopy, a typical apoptotic nuclear pattern was observed (Fig. 2a) with chromatin margination and the occurrence of a promi-

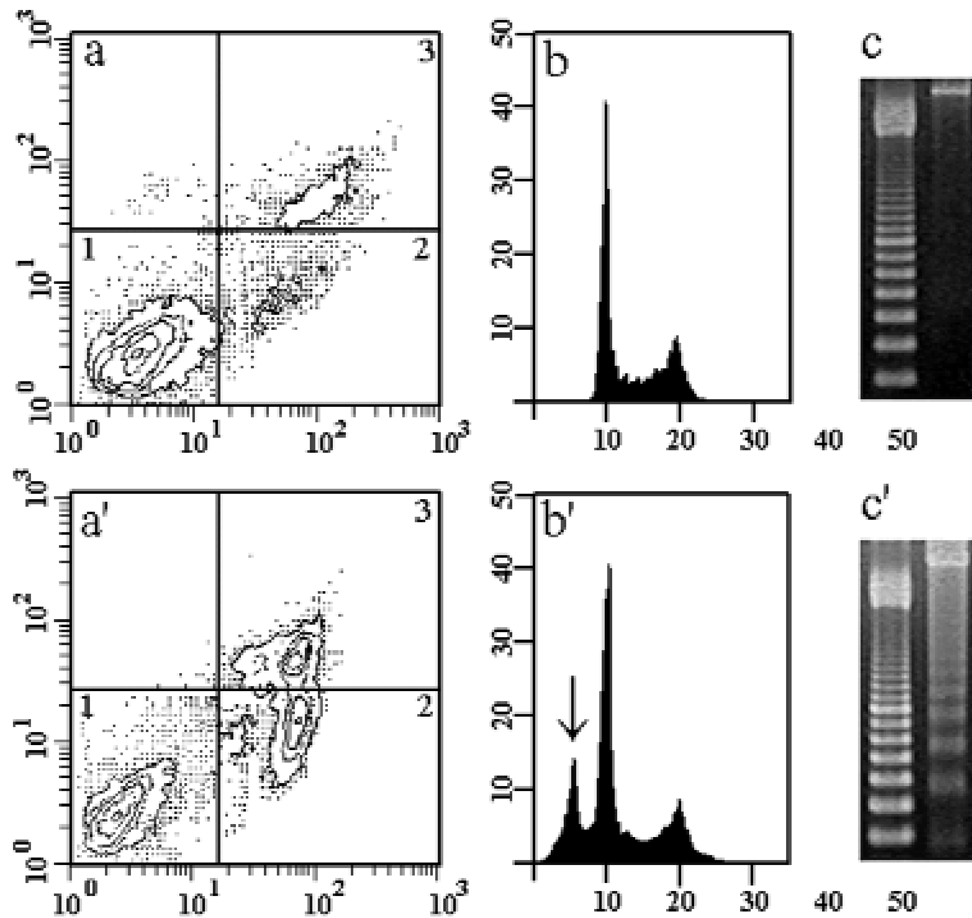


Fig. 1 - a-a') Dual-parameter scattergrams of FITC-labeled Annexin V (in abscissa) versus PI staining (in ordinate) of control (a) and HT-treated EUE cells (a'): the fractions of early (quadrant 2) and late (quadrant 3) apoptotic cells increased in HT conditions (quandrant 1 is for non-apoptotic cells); b-b') PI-DNA content (abscissa, in arbitrary units of fluorescence intensity) for control (b) and HT-treated cells (b'): a prominent sub-G1 peak (arrow) is apparent in HT-treated cells (in ordinate: number of cell x100). c-c') The presence of apoptotic cells after HT treatment (c') was also confirmed by the the typical DNA laddering after agarose gel electrophoresis (the left lane in either panel is for the standard).

nent membrane blebbing. Nuclear RNP containing structures such as PF and PG (which are both located at the periphery of condensed chromatin, in non-apoptotic cells), and IG (which are present in the interchromatin space) undergo severe rearrangement in apoptotic EUE cells: namely, they aggregate into heterogeneous fibrogranular clusters Fig. 2b. The immunolabelings for the antigen Sm and for the hnRNP core proteins are shown in Fig. 2c and 2d, respectively. These RNP containing structures are fully reminiscent of those clusters we described in spontaneously apoptotic thymocytes (Biggiogera *et al.*, 1997a, b) and which we called HERDS (for Heterogeneous Ectopic RNP-Derived Structures: Biggiogera *et al.*, 1998). Consistent with HERDS in apoptotic thymocytes, these clusters may be also found in the cytoplasm of HT-induced apoptotic cells (Fig. 2b).

After HT stress, we also observed a previously unnoticed evidence of restructuring and displacement of RNP-containing structures: in non-apoptotic EUE cells, only the nucleoli were immunolabeled with the anti-fibrillarin antiserum (Fig. 3a,c), whereas after HT stress, when the nucleoli often disaggregated, discrete extra-nuclear immunolabeling for fibrillarin was also found in the cytoplasm (Fig. 3a,d) suggesting that protein aggregates of nucleolar origin may leave the nucleus and migrate into the cytoplasm.

Consistently, at the electron microscope level, nucleolus-like bodies (NLBs) were observed in the cytoplasm. These NLBs (Fig. 4a,b) were comparable, in their morphology, to the functional nucleoli of non-apoptotic EUE cells: namely, the main nucleolar parts (i.e., the dense fibrillar component, fibrillar centers, and the granular component) were recognizable, and no signs of nucleolar segregation were observed. NLBs may also be

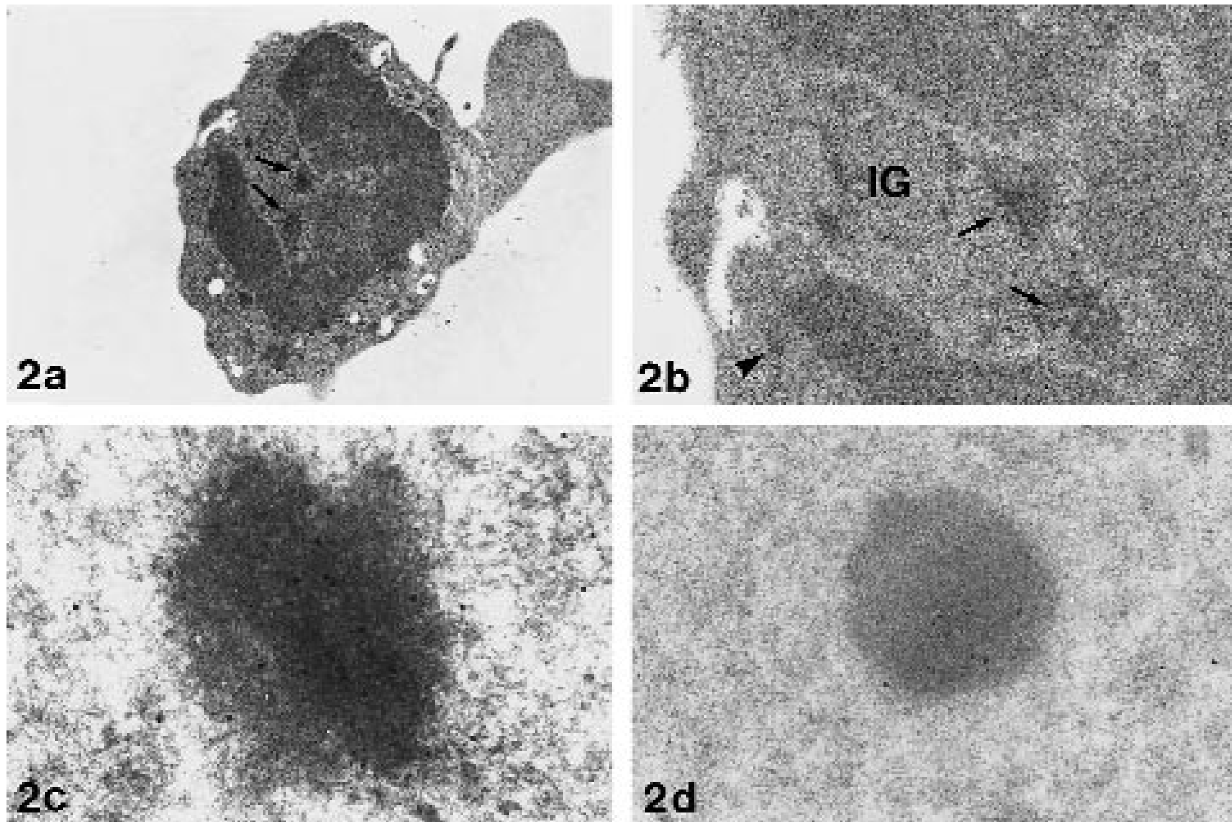


Fig. 2 - Apoptotic EUE cell after HT treatment. a) Marginal chromatin condensation occurred, while in the interchromatin space HERDS were found (arrows). b) Higher magnification of the interchromatin space, where HERDS (arrows) show their fibrogranular and heterogeneous structure, with prominent clusters of interchromatin granules (IG); RNP aggregates of nuclear origin were also found in the cytoplasm (arrowhead). These heterogeneous RNP clusters are immunolabeled for the antigen Sm (c) and for the hnRNP core protein (d).

found inside apoptotic bodies blebbing out at the cell surface of late apoptotic cells (Fig. 4c).

DISCUSSION

Our results confirm that hypertonicity can induce apoptosis in EUE cells, consistent with the results obtained for other cells lines or systems (Anderson *et al.*, 1997; Parolari *et al.*, 1997; Hizoh *et al.*, 1998; Wu *et al.*, 1999). The presence of the typical DNA laddering as well as the microscopical and cytochemical data, suggest that HT-induced apoptosis occurs through a "classic" series of biochemical and morphological events. Moreover, the cytometric evidence of a prominent sub-G1 peak in DNA histograms indicates that apoptosis mainly occurs in G0/1 phase of the cell cycle.

A massive rearrangement of nuclear RNP-containing structures takes also place after HT treatment, with the formation of heterogeneous aggregates first in the interchromatin space and then in the cytoplasm; these aggregates contain at least two antigens which are spatially and/or functionally unrelated in normal cells and may thus be defined as HERDS, according to our definition (Biggiogera *et al.*, 1998; Biggiogera and Pellicciari, 2000).

It has been reported that short-term exposure to HT media induces a significant decrease in protein synthesis (Petronini *et al.*, 1987; Hirata *et al.*, 1987; Pellicciari *et al.*, 1993), mostly due to transcription inhibition. This is in good agreement with the hypothesis that HERDS may generally originate as a consequence of transcriptional arrest (Biggiogera and Pellicciari, 2000).

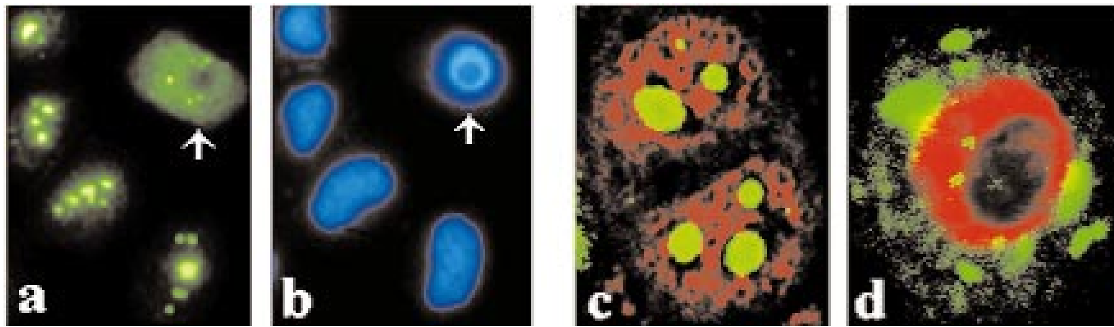


Fig. 3 - a) Fluorescence micrographs showing the FITC-immunolabeling of EUE cells for the nucleolar protein fibrillarin: in non-apoptotic cells, the immunofluorescence signal is located on the nucleoli only, whereas in apoptotic cells (arrow) the labeled material is fragmented and seems to migrate into the cytoplasm; b) the same field after DNA counterstaining with DAPI, which identifies the typical chromatin condensation of the apoptotic cell. The distribution pattern of anti-fibrillarin-positive material (green areas) in non-apoptotic cells (c) and its fragmentation and migration in apoptotic cells (d) was confirmed by confocal microscopy; the nucleus was counterstained for DNA with propidium iodide.

We present here a previously unnoticed phenomenon occurring during induced apoptosis, i.e. the extrusion into the cytoplasm of ectopic NLBs which are sometimes similar in their ultrastructural appearance to the functional nucleoli of non-apoptotic cells. Even in the cytoplasm, NLBs still preserve their ability to react to antisera recognizing nucleolar proteins such as fibrillarin.

This generally high resistance of RNP components of nuclear origin to proteolytic degradation during apoptosis has already been reported (Casiano *et al.*, 1996; Biggiogera *et al.*, 1997b; Pellicciari *et al.*, 1999; Rosen and Casciola Rosen, 1999), and the present results on the immunolabeling of cytoplasmic NLBs suggest that also nucleolar protein moieties may be only partially (or even not) cleaved by caspases.

At late apoptotic stages, HERDS (and even

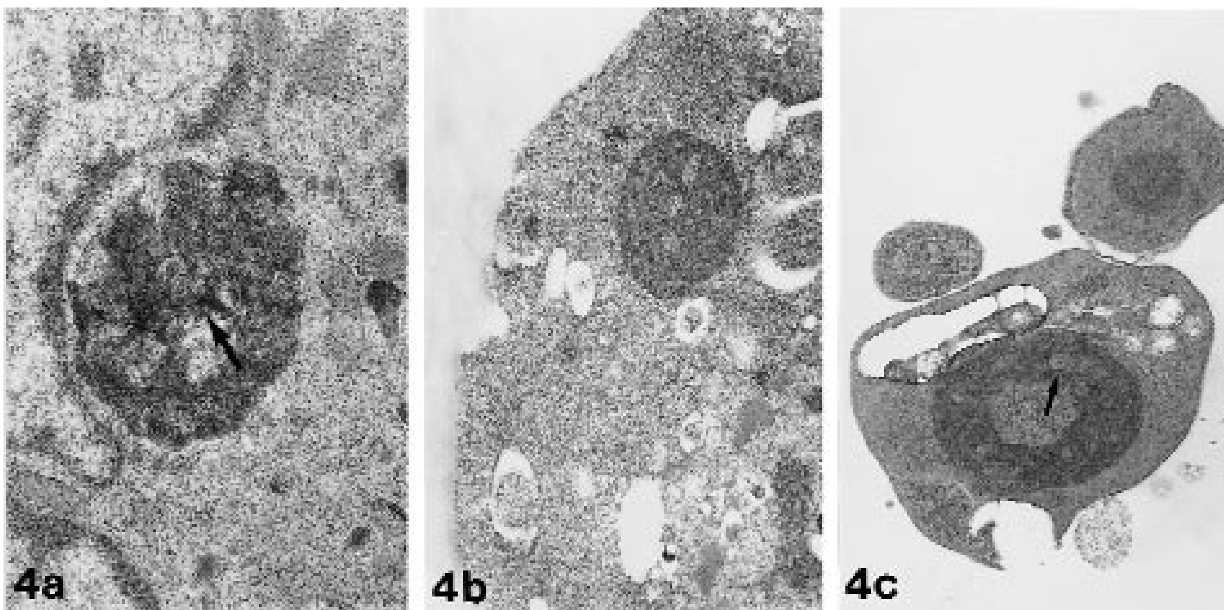


Fig. 4 - Nucleolus-like bodies (NLBs) in the cytoplasm, first in close proximity of the nuclear envelope (a), then approaching the plasma membrane (b). NLBs were sometimes also found inside apoptotic bodies (c). In a), the arrow points to the dense fibrillar component, while in c) it indicates one of the numerous fibrillar centres.

NLBs, as we demonstrate in the present investigation) may be found in the released apoptotic bodies, where the concentration of RNP-containing aggregates can be high. Apoptotic bodies are usually engulfed *in vivo* by phagocytic cells (for a recent review, see Dini, 2000); a defective clearance of undegraded (or partially modified) macromolecular complexes, such as the RNP containing aggregates, may thus be a relevant event for the etiology of autoimmune diseases (Rosen and Casciola-Rosen, 1999; Piacentini, 1999). Actually, the presence of high-titre autoantibodies against antigens of RNP or nucleolar origin is rather common in the serum of patients with autoimmune diseases such as systemic lupus erythematosus (Casciola-Rosen *et al.*, 1994; Casiano *et al.*, 1996).

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