Journal of Cellular Biochemistry 78:264–277 (2000)

Behavior of Nucleolar Proteins During the Course of Apoptosis in Camptothecin-Treated HL60 Cells

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Abstract By means of immunofluorescence and immunoelectron microscopy we have studied the fate of different nucleolar components during the apoptotic process in camptothecin-treated HL60 cells. We have found that RNA polymerase I disappeared while UBF was associated with previously described fibrogranular threaded bodies. In contrast, fibrillarin, C23/nucleolin, and B23/nucleophosmin remained detectable in granular material present amid micronuclei of late apoptotic cells. Double immunolabeling experiments showed colocalization of both C23 and B23 with fibrillarin. Immunoblotting analysis showed that UBF was proteolytically degraded, whereas fibrillarin, C23/nucleolin, and B23/nucleophosmin were not. These results may help explain the presence of anti-nucleolar antibodies seen in various pathological disorders. J. Cell. Biochem. 78:264–277, 2000.

Key words: Keywords: apoptosis; nucleolus; HL60 cell; proteins; immufluorescence; immunoelectron microscopy

Apoptosis is a highly regulated process leading to cell death. It is largely conserved through evolution and may occur in response to a diverse range of stimuli. Its significance is underscored by its occurrence in all higher eukaryotes and by malformations resulting from disturbances of the apoptotic process during development [Vaux and Korsmeyer, 1999]. Increased rates of apoptosis are responsible for several degenerative diseases, and inhibition of apoptosis is implicated in autoimmune processes and carcinogenesis [Bellamy et al., 1995; Hale et al., 1996; Vaux and Strasser, 1996; Jacobson et al., 1997]. Among central components of the apoptotic machinery are most likely proteases, nucleases, phosphatases, and kinases, which are responsible for the biochemical and morphological changes peculiar to the apoptotic process [Hale et al., 1996; Vaux and Strasser, 1996; Villa et al., 1997]. After commitment of cells to apoptosis, characteristic morphological changes are observed in the execution phase of cell death. These alterations include cytoplasmic condensation and plasma membrane blebbing, cell shrinkage, condensation of chromatin that marginates towards the inner nuclear membrane forming cap-shaped, compact areas, and, ultimately, fragmentation of residual nuclear structures into apoptotic bodies that are phagocytosed by neighboring cells and macrophages [Hale et al., 1996; Vaux and Strasser, 1996; Falcieri et al., 1994; Earnshaw, 1995; Kroemer et al., 1995; Dini et al., 1995; Martelli et al., 1997; Renò et al., 1998]. Most likely, these changes reflect proteolysis of several nuclear and cytoplasmic polypeptides, and DNA degradation by endogenous nucleases into at first large and then finally nucleosomal-size fragments [e.g., Collins et al., 1997].

We, and others, have recently demonstrated that during the apoptotic process fibrogranular threaded bodies, containing ribonucleopro-

Grant sponsor: Associazione Italiana per la Ricerca sul Cancro (AIRC); Grant sponsor: Italian MURST; Grant sponsor: Cofinanziamento MURST 1998 and 1999.

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Received 13 October 1999; Accepted 27 January 2000

Print compilation © 2000 Wiley-Liss, Inc.
This article published online in Wiley InterScience, May 2000.

teins, RNA, and nuclear matrix polypeptides, appear within the nucleus and are subsequently extruded to be released at the cell surface [Zweyer et al., 1995, 1997; Lafarga et al., 1997; Biggiogera et al., 1997a,b, 1998]. Although these bodies resemble coiled bodies, they do not contain either fibrillarin or p80 coilin [Zweyer et al., 1995], i.e., two proteins that are recognized markers of this class of nuclear inclusions [Ochs et al., 1994; Lamond and Earnshaw, 1998; Schul et al., 1998]. Apparently, these bodies originate from the nucleolus, but it is still unclear from which nucleolar domain(s) they come. With the above in mind, we undertook this study in order to understand the exact nucleolar origin of these distinctive nuclear inclusions, using antibodies that are specific for proteins of the various nucleolar subcompartments. Moreover, since the nucleolus is considered to be very resistant to apoptotic cell death [Columbaro et al., 1999], we sought to determine the behavior, during the apoptotic process, of other proteins that are peculiar to the various nucleolar subcompartments.

MATERIALS AND METHODS

Cell Culture and Induction of Apoptosis

HL60 human promyelocytic leukemia cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. To induce apoptosis, cells were synchronized at the G1/S border with 1 μg/ml aphidicolin for 15 h. They were then washed three times in RPMI-1640 to remove aphidicolin. Aliquots of cells at this stage were used as control cells. For induction of apoptosis, cells were exposed up to 3 h to 0.1 μg/ml of the DNA topoisomerase I inhibitor, camptothecin (Sigma Chemical Co.), according to Zweyer et al. [1997].

Antibodies

The following antibodies to nucleolar components were employed in the present study: S4, a human autoantibody to fibrillarin, which is found in the dense fibrillar component; J.O., a human autoantibody to NOR-90/UBF, which localizes to fibrillar centers and dense fibrillar component; Pol I, a human autoantibody to RNA polymerase I (RNA pol I), which also labels fibrillar centers; MS3, a mouse monoclonal antibody (IgG) to protein C23/nucleolin, which mainly immunolabels the granular component; MB23, a mouse monoclonal antibody (IgG) to protein B23/nucleophosmin, which mainly localizes to the granular component. For general references about these antibodies, see Ochs [1998]. Monoclonal antibody to PARP (clone C-2-10) was from Oncogene Research Products.

Immunofluorescent Staining

The procedure was carried out essentially as described by Neri et al. [1997]. Briefly, cells in media were plated onto 0.1% poly-L-lysine-coated glass slides and adhesion was allowed to proceed for 30 min at room temperature. Samples were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature and then permeabilized with 0.2% Triton X-100 for 10 min. After several washes with PBS, nonspecific binding of antibodies was blocked by a 30-min incubation at 37°C with PBS, 2% bovine serum albumin (BSA), 5% normal goat serum (NGS). Slides were then incubated for 3 h at 37°C with the appropriate primary antibodies (all diluted 1:1,000) in PBS, 2% BSA, 5% NGS. Slides were then washed three times in PBS and reacted with Cy2-conjugated anti-mouse IgG or anti-human IgG (Jackson Immunoresearch Lab, Inc.), diluted 1:100 in PBS, 2% BSA, 5% NGS for 1 h at 37°C. For double labeling experiments, human antisera and monoclonal antibodies were revealed by means of Cy2-conjugated anti-human IgG and Cy3-conjugated anti-mouse IgG (Sigma), respectively. Samples were subsequently washed three times in PBS, stained with 0.01 μg/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS and mounted in 20 mM Tris-HCl, pH 8.2, 90% glycerol containing 2.3% of the antifading agent 1,4-diazobicyclo-[2.2.2]-octane. Slides were observed and photographed using a Zeiss Axiophot epifluorescence microscope.

Immunogold Labeling and Electron Microscopy

Cells were fixed in 4% freshly-prepared paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h and washed twice in phosphate buffer. Samples were dehydrated sequentially in 50, 75, and 90% N, N dimethyl-formamide and embedded at 4°C in Lowicryl K4 M followed by UV polymerization [Falcieri et al., 1994]. The sections were collected on 200-mesh nickel grids and incubated for 10 min at room temperature in TBS blocking buffer (50 mM Tris-HCl, pH 7.6, 1% sodium azide, 150 mM

NaCl, 0.1% BSA). The grids were treated with 5% NGS for 30 min at room temperature, and incubated overnight at 4°C in the presence of the primary antibodies, used at the following dilutions: 1:100 for J.O., Pol I, and MS3; 1:300 for MB23. After several washes in TBS buffer, the samples were incubated in TBS buffer (pH 8.2) for 1 h at room temperature with 15 nm gold-conjugated goat anti-human IgG (British Biocell International, diluted 1:10, for J.O. and Pol I), and with 10 nm gold-conjugated goat anti-mouse IgG (diluted 1:8 for MB23 and MS3). After three washes in TBS (pH 8.2) and one in distilled water, the grids were stained with uranyl acetate and observed with a Zeiss 109 electron microscope. Controls were represented by samples treated with the secondary antibodies alone: no gold particles were detected (data not shown).

For conventional electron microscopy, samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and processed as previously described [Falcieri et al., 1994].

Polyacrylamide Gel Electrophoresis and Immunoblotting of Cell Lysates

Cells were sedimented at 1,000*g* for 10 min and washed twice in PBS containing the COMPLETE Protease Inhibitor Cocktail (Roche Molecular Biochemicals), according to the manufacturer's instructions. Cells were then resuspended at $\sim 10^7$/ml in lysis buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecylsulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and the protease inhibitor cocktail. Lysates were boiled for 5 min to solubilize protein, passed several times through a 27-gauge needle to shear DNA, and stored at −80°C until required. Cell harvesting and lysate preparation were conducted in the presence of the protease inhibitor cocktail as a precaution to prevent further proteolysis. Total protein from $\sim 5 \times 10^6$ cells was separated by SDS-polyacrylamide gel electrophoresis [Laemmli, 1970] and electrophoretically transferred to nitrocellulose sheets using a semi-dry blotting apparatus (Hoefer/Pharmacia Biotech). Sheets were saturated in PBS containing 5% NGS and 4% BSA for 60 min at 37°C (blocking buffer), then incubated overnight at 4°C in blocking buffer containing the primary antibodies diluted 1:1,000. After four washes in PBS containing 0.1% Tween 20, they were incubated for 30 min at room temperature with peroxidase-conjugated anti-mouse or anti-human IgG (from Sigma), diluted 1:3,000 in PBS-Tween 20, and washed as above. Bands were visualized by the enhanced chemiluminescence method using Lumi-Light^{Plus} (Roche Molecular Biochemicals).

RESULTS

Conventional Electron Microscope Analysis

In Figure 1a we show the ultrastructural morphology of the nucleus of a control HL60 cell. The nucleus was convoluted, as is typical for these cells.

It should be emphasized that samples representative of the early apoptotic stages were seen after a 60–90-min exposure to camptothecin, while those displaying features characteristic of the late apoptotic stages were evident after a 150–180-min incubation in the presence of the drug. HL60 cells exposed to camptothecin displayed the characteristic nuclear changes of the apoptotic process. In the early stages, chromatin marginated towards the nuclear periphery, and appeared sharply separated from more diffuse chromatin (Fig. 1b). However, already at this time 0.5–1 μm nuclear bodies composed of ribonucleoprotein were evident, as well as nucleoli mostly characterized by component segregation. In the late stages of the execution phase, typical micronuclei were observed, scattered throughout the cytoplasm (Fig. 1c).

Immunofluorescence

Autoantibody to fibrillarin homogeneously stained nucleoli in control cells, as evidentiated also by DAPI staining and phase-contrast microscopy (Fig. 2A). The immunoreactivity was maintained in apoptotic samples, both when cap-shaped chromatin marginations were observed (Fig. 2B) and when micronuclei were present (Fig. 2C).

Also, human anti-UBF serum selectively stained nucleoli (Fig. 2A). Some very faint immunoreactivity was seen at early (Fig. 2B) but not at late stages (Fig. 2C) of the apoptotic process, in the form of very small dots with a diameter of approximately 0.5 μm.

The antibody to RNA pol I selectively immunodecorated nucleoli (Fig. 2A). In apoptotic cells, the immunoreactivity almost disappeared by the time cap-shaped marginations were detected, even though some very small dots were observed (Fig. 2B). No immunostaining was present at later stages (Fig. 2C).

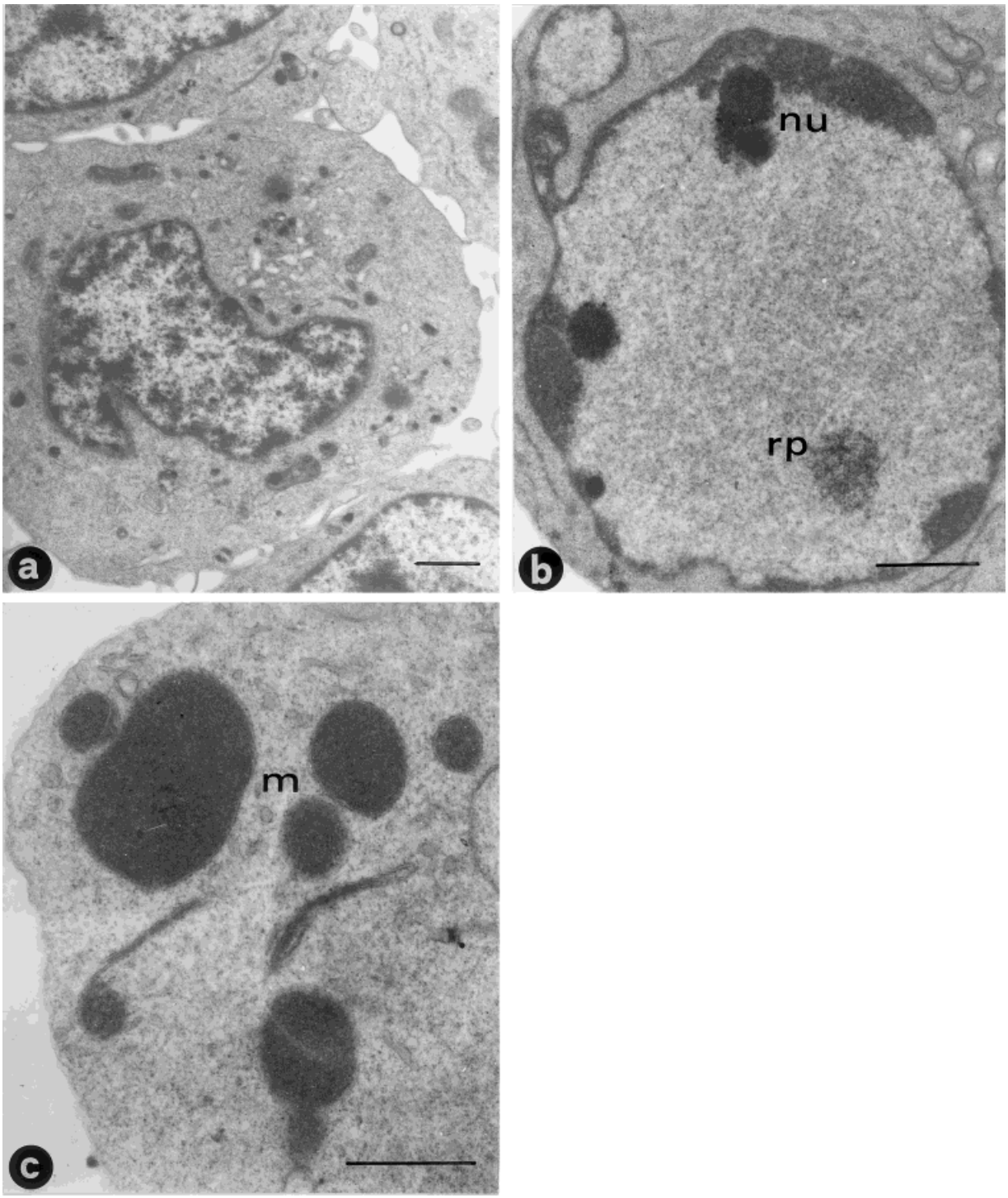

Fig. 1. Conventional electron microscopy of HL60 cells. Control cell (**a**), early (**b**), and late (**c**) apoptotic cell. nu = nucleoli, rp = ribonucleoprotein granules, m = micronuclei. Scale bar = 1 μm.

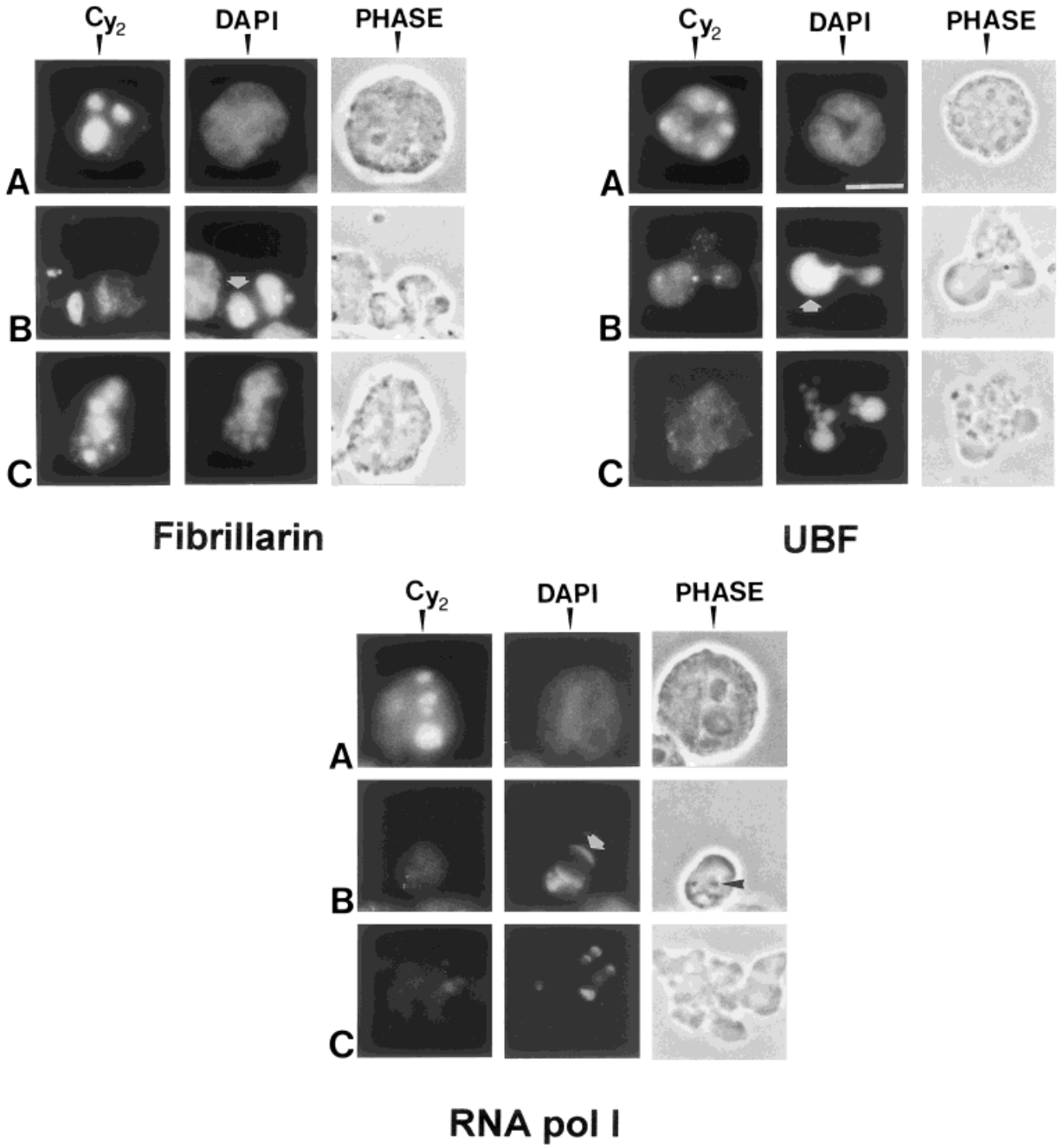

Fig. 2. Immunofluorescent staining for fibrillarin, UBF and RNA pol I. **A:** Control cells. **B,C:** Apoptotic cells. The white arrow indicates nuclei with cap-shaped chromatin marginations. The black arrowhead points to a nucleolus that is capped by chromatin marginations. Scale bar = 5 μm.

The nucleolar staining pattern given by monoclonal antibody to protein B23 was homogeneous (Fig. 3A). This pattern was also apparently maintained throughout the various phases of apoptotic nuclear destruction (Fig. 3B,C). However, the immunoreactivity particularly concentrated at the cell periphery at the late stages of apoptosis (Fig. 3C).

The monoclonal antibody to protein C23 was specific for nucleoli in control cells (Fig. 3A). The staining was apparently maintained in all the stages of apoptotic nuclear changes (Fig.

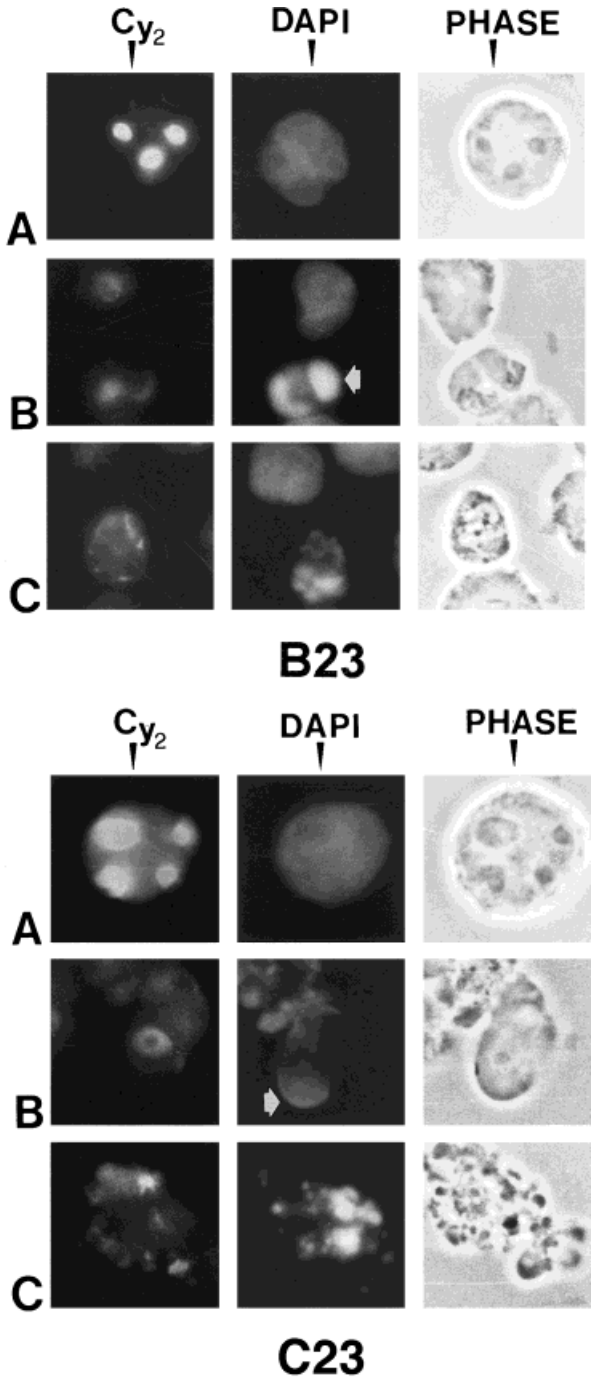

Fig. 3. Immunofluorescent staining for B23 and C23 proteins. **A:** Control cells. **B,C:** Apoptotic cells. The white arrowhead indicates nuclei with cap-shaped chromatin marginations. Scale bar = 5 μm.

3B,C), even though at the late stage the immunoreactivity was detectable at the periphery of the apoptotic bodies (Fig. 3B).

In Table I we report, for each of the antigens studied, the percentage of apoptotic samples in which we detected the immunofluorescent pattern illustrated in the pictures. It is evident that the pattern we have chosen as "representative" was seen in the majority of apoptotic cells.

We also performed double immunolabeling for both C23 and B23 with fibrillarin. As shown in Figure 4 we saw a colocalization of the two signals at the late stages of the apoptotic process.

Immunogold Labeling

Anti-UBF serum specifically stained nucleolar fibrillar centers of control cells (Fig. 5a). In apoptotic cells, the immunolabeling was mainly localized to the dense fibrillar component, which was clearly segregated from the granular component (Fig. 5b). Ribonucleoprotein granules, observable within apoptotic diffuse chromatin, were labeled by the antiserum (Fig. 5c). In late apoptotic cells, positively labeled clusters of ribonucleoproteins were still recognizable within micronuclei (Fig. 5d).

Antiserum to RNA pol I mostly labeled nucleoli in normal cells (Fig. 6a). In apoptotic cells, where the segregation of fibrillar and granular component occurred, the immunogold labeling disappeared, in agreement with immunofluorescence data (Fig. 6b).

Monoclonal antibody to nucleolin mostly stained the granular component of non-apoptotic nucleoli, whereas fibrillar centers appeared consistently negative (Fig. 7a). The positivity was well maintained in nucleoli of apoptotic cells (Fig. 7b). In apoptotic nucleoli showing the segregation of fibrillar and granular components, a strong labeling was observed on the latter, even though some gold particles also appeared in the fibrillar component (Fig. 7c). In late apoptotic cells, we observed a specific immunogold labeling on cytoplasmic granular material, clustered between micronuclei and ribonucleoprotein granules, which were always negative (Fig. 7d).

Finally, monoclonal antibody to B23 protein gave a strong positivity for the granular component of non-apoptotic nucleoli (Fig. 8a), whereas fibrillar centers were mostly negative. In apoptotic cells, the immunolabeling was still selective for nucleoli. Nucleolar-associated chromatin and ribonucleoprotein granules were negative (Fig. 8b). In apoptotic nucleoli displaying a segregation into fibrillar and granular components, we detected a specific positivity in the latter (Fig. 8c). In late apopto-

TABLE I. Percentage of Apoptotic Samples in Which We Detected the Typical Immunofluorescent Pattern Illustrated in the Pictures*

	Fibrillarin	UBF	RNA pol I	B23	C23
Early apoptotic stages	68.8 ± 9.7	74.6 ± 10.2	79.6 ± 9.9	64.8 ± 8.6	73.3 ± 8.8
Late apoptotic stages	77.4 ± 9.3	83.6 ± 8.5	78.4 ± 9.1	76.9 ± 9.0	89.7 ± 10.1

*Data are the mean from three different experiments ± SD. 100 apoptotic cells for experiment were manually counted and examined.

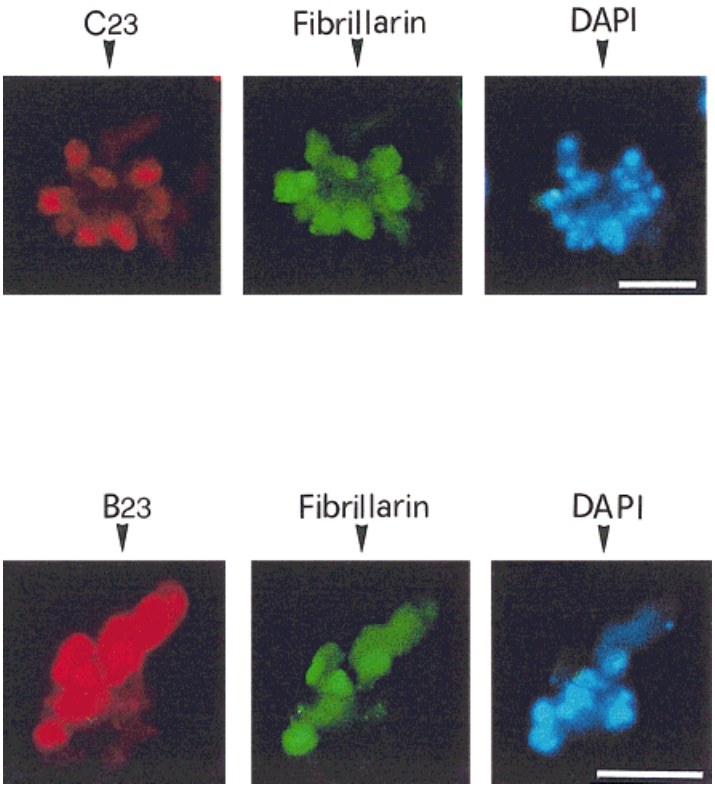

Fig. 4. Double immunofluorescent staining for either C23 or B23 protein and fibrillarin. A late stage of the apoptotic process (note the micronuclei stained by DAPI) is shown. Scale bar = 5 μm.

tic stages, a strongly positive cytoplasmic material was still present within micronuclei (Fig. 8d).

Immunoblotting Analysis

By immunoblotting analysis, we sought to determine whether or not the nucleolar proteins that we have studied in our investigation were proteolysed. We prepared extracts from control and apoptotic cells (after 3 h exposure to camptothecin, i.e., a time by which DAPI staining revealed that approximately 70% of the cells were apoptotic, with a prevalence of the most advanced stages, data not presented), which were then electrophoresed, blotted to nitrocellulose paper, and probed with the primary antibodies. As a control marker of proteolytic degradation, we employed a monoclonal antibody to PARP, i.e., a protein that is cleaved during the apoptotic process [see for example Casiano et al., 1996; Scovassi et al., 1998]. As shown in Figure 9, in control cells the antibody to PARP recognized a protein with an apparent Mr of 115 kDa. In apoptotic cells, the amount of the native protein decreased, whereas a fragment migrating at approximately 85 kDa became apparent. Since antiserum to RNA pol I did not work for immunoblotting analysis, we restricted our study to fibrillarin, UBF, B23, and C23. As shown in Figure 9, anti-UBF re-

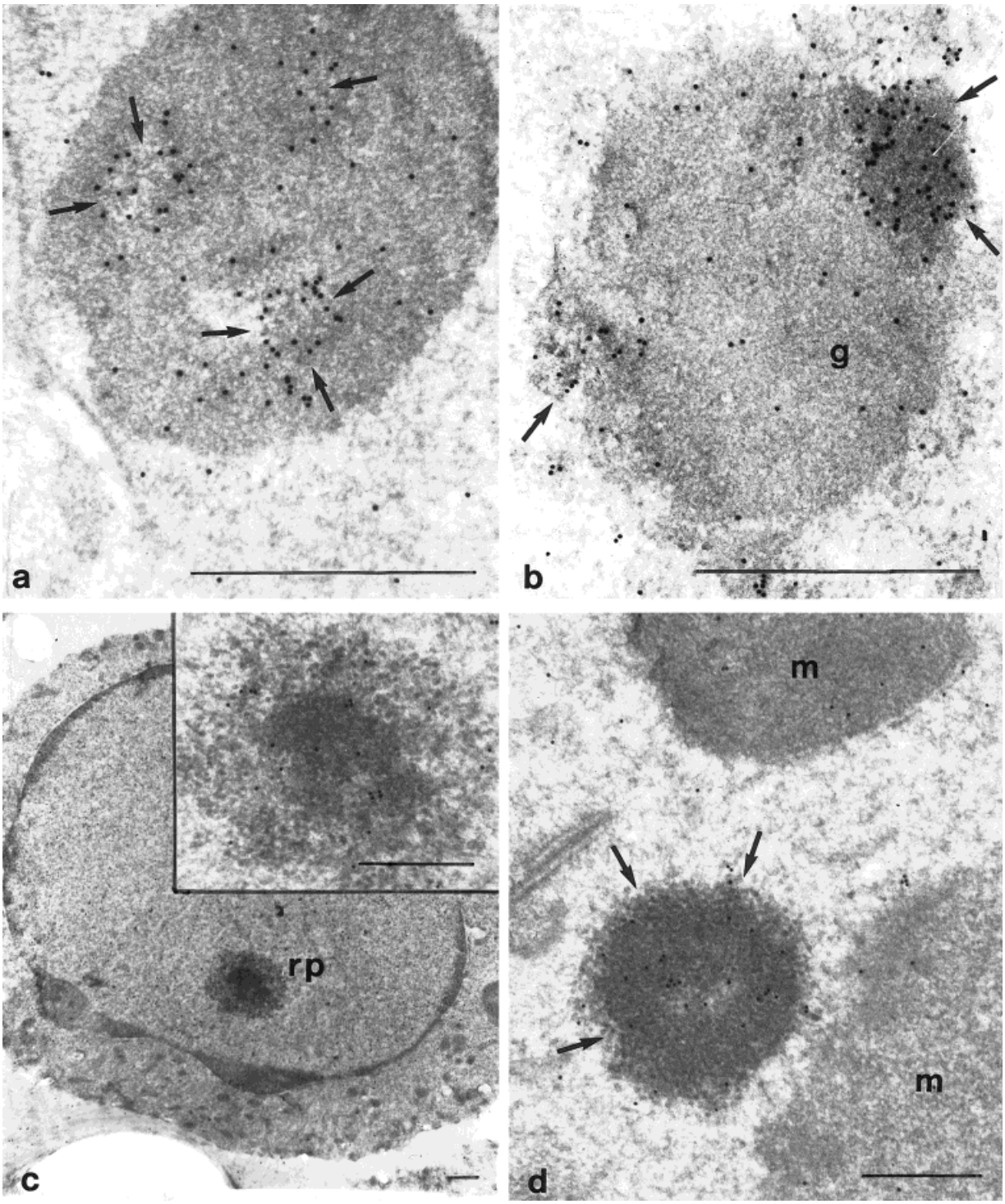

Fig. 5. Immunogold localization of UBF. **a:** The nucleolar positivity, with particular specificity on nucleolar fibrillar centers, is shown in a control cell (arrows). **b:** nucleolar labeling in an apoptotic cell, particularly localized on dense fibrillar component (arrows), visibly segregated from the granular component (g). **c:** Ribonucleoprotein granules (rp), clearly observable within apoptotic diffuse chromatin, are positively labeled (**inset**). **d:** in late apoptotic cells, positive clusters of ribonucleoproteins (arrows) are still recognizable in cells with micronuclei (m). Scale bar = 1 μm.

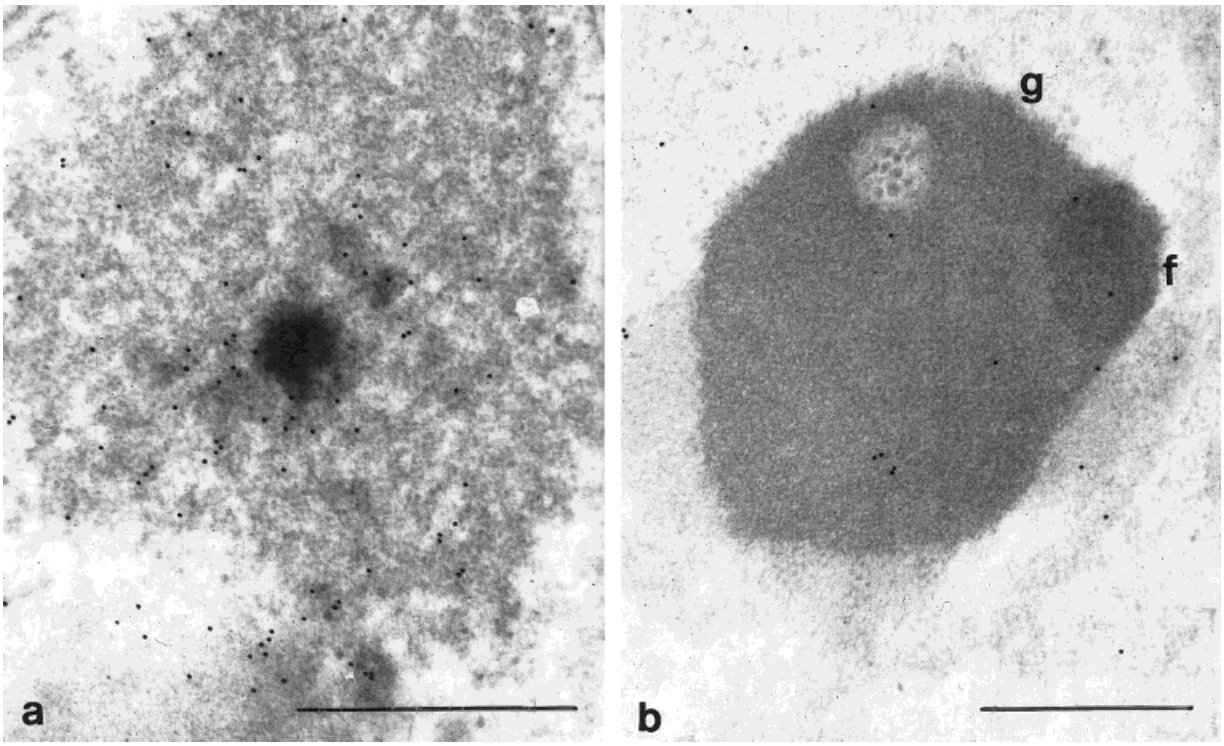

Fig. 6. Immunolocalization of RNA pol I. **a:** Normal cells show mostly labeled nucleoli. **b:** In apoptotic cells, where the segregation of filamentous (f) and granular component (g) occurs, the immunogold labeling disappears. Scale bar = 1 μm.

vealed the existence in apoptotic cells of multiple fragments, a clear indication of proteolysis. On the contrary, the other three antigens did not show any sign of proteolytic cleavage.

DISCUSSION

The morphological changes typical of apoptotic nuclear destruction have long been recognized. They consist of chromatin condensation and margination, changes in the distribution of nuclear pores, nucleolar segregation, and formation of micronuclei [e.g., Falcieri et al., 1994]. More recently, however, several investigators have described the occurrence in the nucleus of apoptotic cells of heterogeneous fibrogranular clusters that resemble coiled bodies [Zweyer et al., 1997; Lafarga et al., 1997]. These clusters apparently originate from the nucleolus, but they also contain ribonucleoprotein derived from perichromatin fibrils, interchromatin and perichromatin granules, as well as RNA, and have been named HERDS (for heterogeneous ectopic ribonucleoprotein-derived structures) [Biggiogera et al., 1997a,b, 1998]. Moreover, these bodies are enriched in several proteins of the insoluble nuclear matrix [Zweyer et al., 1997]. Since it was still unclear the nucleolar domain(s) from which these bodies originated, we undertook the present study using antibodies that are specific for different nucleolar domains.

First, we tested these antibodies by immunofluorescent staining. Our results demonstrated that, with the exception of RNA pol I and, to a lower extent, UBF, the immunoreactivity towards the antigens was quite well maintained even at late stages of apoptotic nuclear destruction up to when micronuclei started to disperse. We have previously observed a similar phenomenon by means of a monoclonal antibody to DNA topoisomerase IIβ, which selectively immunodecorated nucleoli [Zweyer et al., 1995]. This may appear surprising because at the stage of micronuclei, only nucleolar residues could be identified by electron microscopy and this prompted us to analyze the phenomenon in more depth by immunogold labeling.

The use of immunoelectron microscopy allowed us to determine that the granular and threaded bodies were positive for anti-UBF,

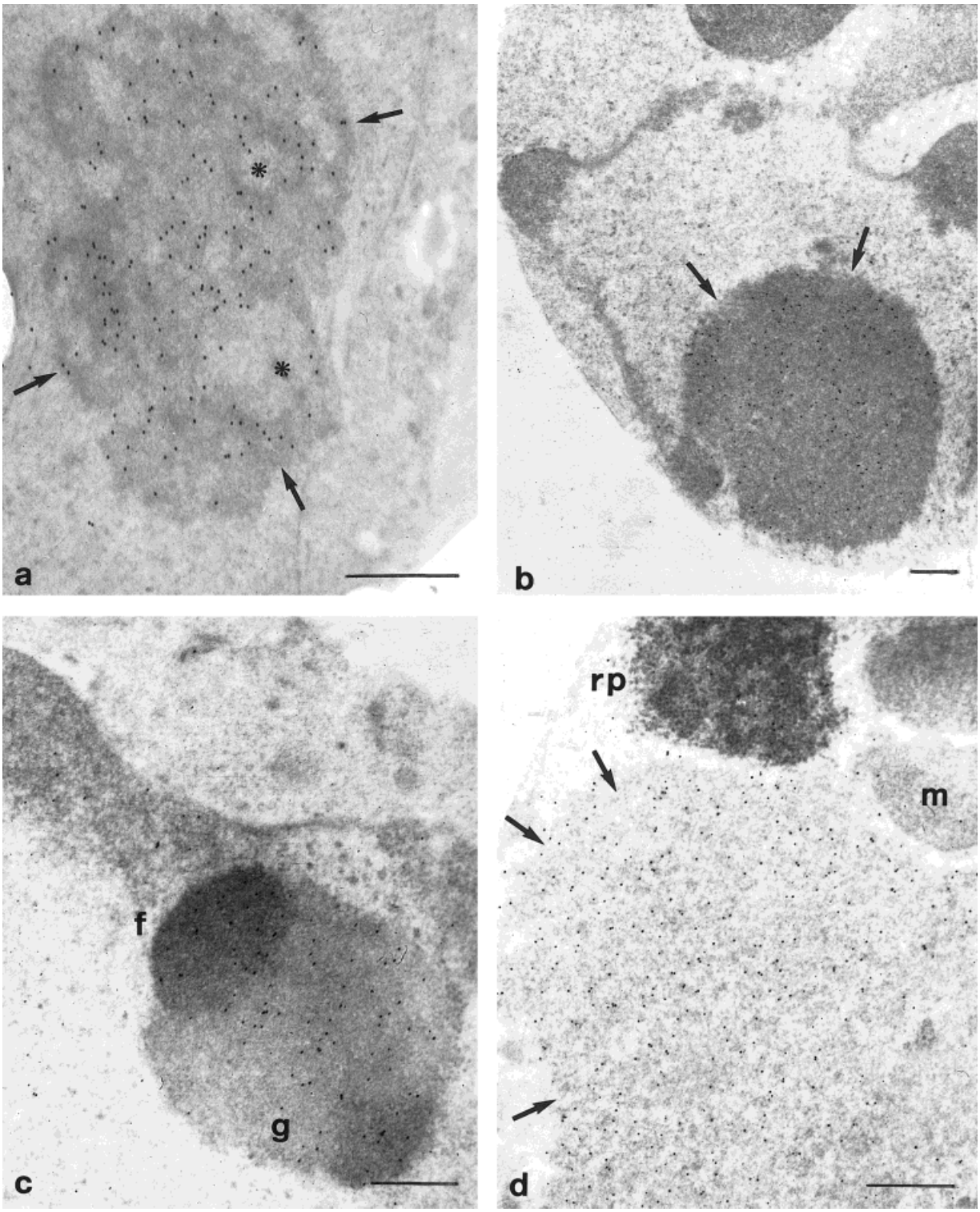

Fig. 7. Immunogold labeling of C23/nucleolin. **a:** Control nucleoli appear selectively positive, mainly on the granular component (arrows). Fibrillar centers appear consistently negative (asterisks). **b:** Nucleolar positivity in an apoptotic cell (arrows). **c:** Apoptotic nucleolus, showing the segregation of fibrillar (f) and granular (g) components, as well as a strong labeling on the latter. **d:** Late apoptotic cells show specific immunogold labeling on cytoplasmic granular material in the proximity of the plasma membrane (arrows), in cells containing micronuclei (m) and ribonucleoprotein granules (rp). Scale bar = 1 μm.

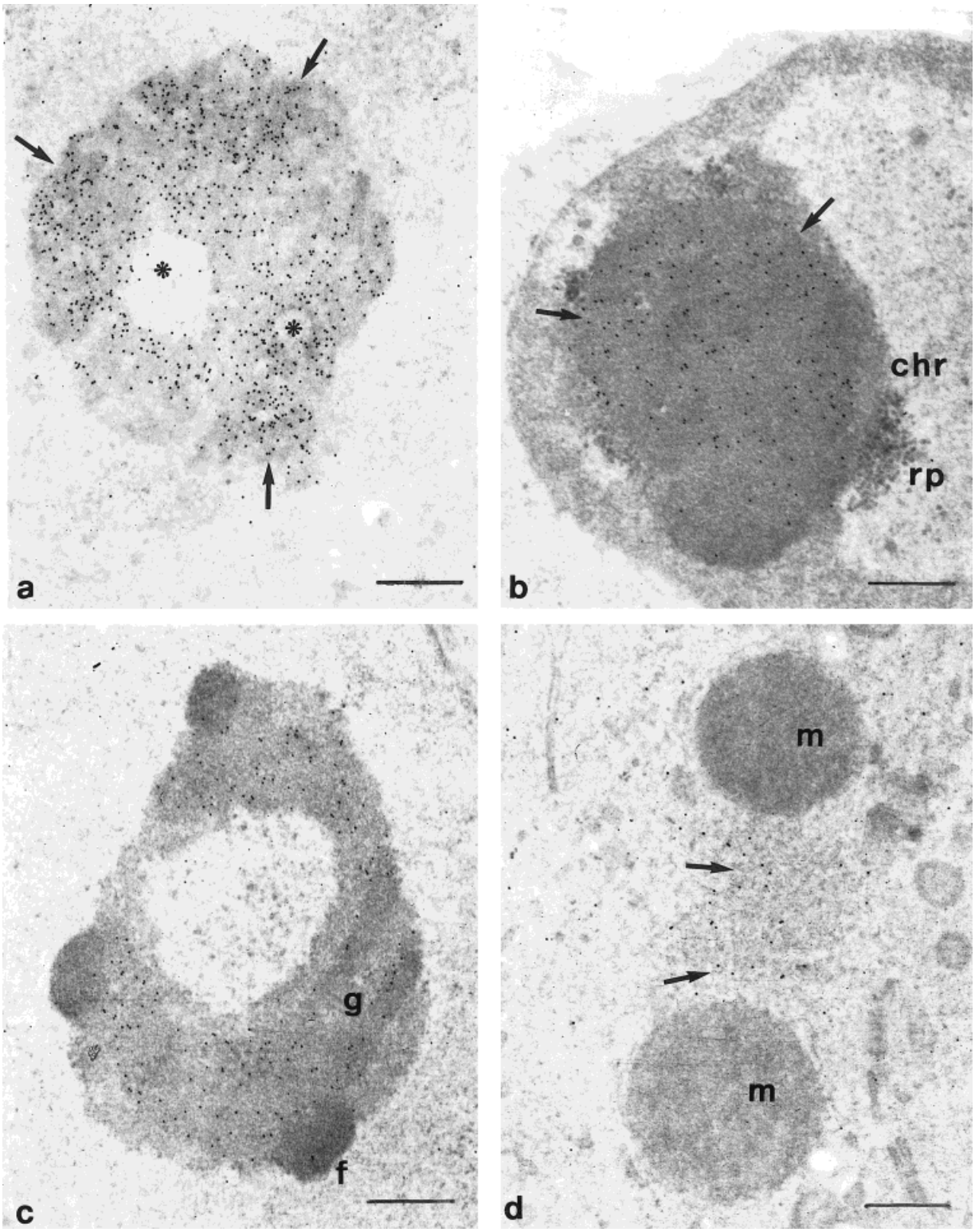

Fig. 8. Immunogold localization of B23/nucleophosmin. **a:** Control cells show a strong positivity of nucleolar granular component (arrows) and mostly negative fibrillar centers (asterisks). **b:** Apoptotic cells present selectively labeled nucleoli (arrows). Nucleolar associated chromatin (chr) and ribonucleoprotein granules (rp) are negative. **c:** Nucleoli segregated in fibrillar (f) and granular (g) components show a specific positivity on the latter. **d:** In late apoptosis, a strongly positive cytoplasmic material (arrows) is still present within cells that contain micronuclei (m) Scale bar = 1 μm.

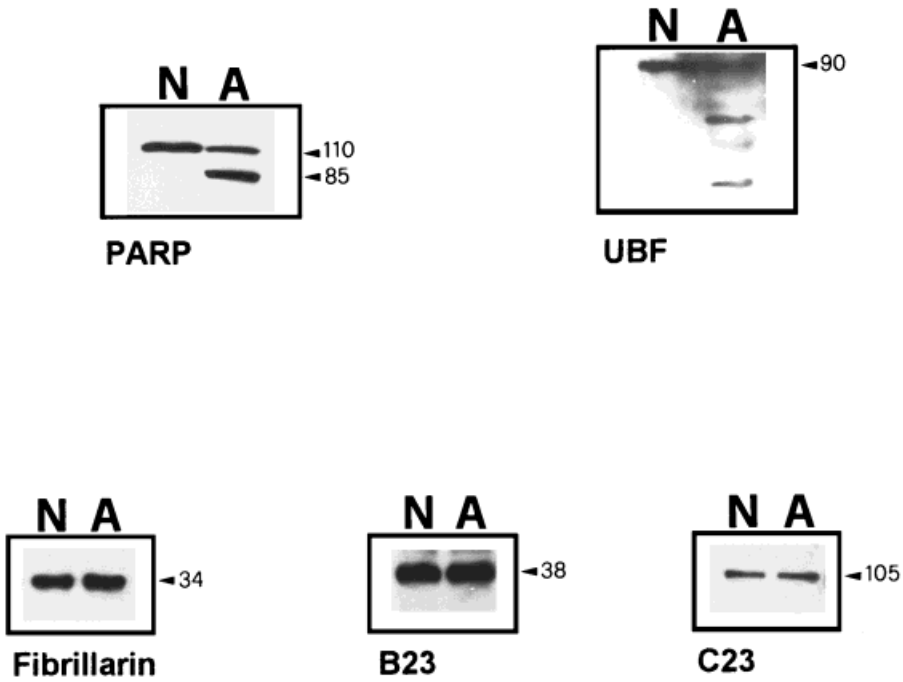

Fig. 9. Immunoblotting analysis for PARP, UBF, fibrillarin, B23, and C23 in normal (N) and apoptotic (A) HL60 cells. The apparent molecular mass of the native protein is indicated at the right. For PARP, the apparent molecular mass of the proteolytic fragment is also shown.

but not for antibodies to RNA pol I, C23, or B23. Since our previous results have shown that these bodies are negative for antibodies to fibrillarin [Zweyer et al., 1995], it is likely that the nucleolar component(s) present in these inclusions derive from the fibrillar centers of the nucleolus. In this connection, it should be recalled that Biggiogera et al. [1998] sometimes found in their HERDS RNA of nucleolar origin, constituted of remnants of the preribosomal fraction deriving from the nucleolar granular component. It should be emphasized that by immunofluorescent staining, we detected UBF in small dots with a diameter of approximately 0.7 μm, that conceivably correspond to the HERDS identified by transmission electron microscopy, whose labeled central area displays a diameter of approximately 0.8–0.9 μm. Interestingly, Biggiogera et al. [1999] have also very recently identified HERDS-like bodies in rat erythroblasts undergoing spontaneous differentiation. The authors have hypothesized that formation of HERDS might be in relationship with a transcriptional arrest.

By immunogold labeling, we also observed that antibodies to C23 and B23 strongly stained material present within micronuclei, which likely corresponds to residues of the granular nucleolar component. Therefore, even if C23 and B23 are not part of the fibrogranular threaded bodies, they are still associated with some remnants of the nucleolar granular component that, once liberated in the very late stages of the process, could constitute material able to stimulate the immune system to produce antibodies against it, in agreement with the most recent hypothesis about the origin of antinuclear autoantibodies [van Venrooij and Pruijn, 1995; Rosen and Casciola-Rosen, 1999]. In fact, the occurrence of autoantibodies against B23 has been reported [Ochs, 1998]. Since these proteins maintain their immunoreactivity, this could be a way by which the immunoreactive material stimulates the immune system to produce antinuclear antibody, which are raised against normally sequestered antigens [Casiano and Tan, 1996; Miranda et al., 1998]. It should be emphasized that the granular material immunolabeled by antibody MS3 was found close to the plasma membrane, and this may indicate that it will eventually be released from the dying cells. The double labeling immunofluorescence experiments allowed us to determine that fibrillarin colocalized with

both C23 and B23. Therefore, this could also help explain the genesis of anti-fibrillarin autoantibodies. B23 and C23 are the two main argyrophilic proteins of the nucleolus [Hozak et al., 1992]. B23 is a phosphoprotein that is involved in the assembly of preribosomes and is also engaged in nucleocytoplasmic shuttling [Zatepsina et al., 1999]. As far as nucleolin is concerned, this protein has long been implicated in rRNA transcription and maturation, as well as in other numerous functions [see Ginisty et al., 1999, for an updated review on nucleolin]. It may be interesting to recall that Chan and Chan [1999] have very recently observed B23 to shift its location from nucleolus to nucleoplasm in apoptotic K562 and HeLa cells.

The fact that RNA pol I immunoreactivity was not observed in samples at the late stages of the apoptotic process, might suggest that the enzyme is degraded and inactivated. Unfortunately, due to the fact that our antibody does not recognize RNA pol I on immunoblots, we could not assess whether or not this protein was actually cleaved.

As far as the other proteins are concerned, UBF was cleaved, whereas both fibrillarin and B23 were not, in agreement with the data by Casiano et al. [1996]. Also, C23 did not undergo proteolytic cleavage, in agreement with our own recent findings [Martelli et al., 1999]. The fact that UBF is cleaved during apoptosis might be related to the recent data by Stegh et al. [1998] who described a novel 37-kDa protein, DEDD, which is situated on the CD95 (Fas /APO-1) signaling pathway for apotosis. Upon Fas stimulation, DEDD translocates to the nucleolus in a manner that is partly dependent on activation of caspases. In the nucleolus, it colocalizes with UBF (where it may facilitate UBF cleavage) and in a reconstituted in vitro system DEDD inhibits rDNA transcription. It should be pointed out that UBF is one of the two factors that are required for accurate in vitro initiation of ribosomal gene transcription by RNA pol I [Bell et al., 1988].

In some apoptotic models, such as thymocytes or other lymphoid cells, nucleolar components can be recognized for a long time in the course of apoptosis. Even by means of conventional ultrastructural staining, granular and fibrillar components can be identified, closely associated with each other or scattered throughout the cytosol [Falcieri et al., 1993, 1994]. In other cell lineages undergoing apoptosis, nucleoli seem to progressively disappear and only rare nucleolar structures can be revealed in late apoptotic stages [Columbaro et al., 1999]. However, the immunostaining techniques employed in our study demonstrated that the granular material frequently observed in proximity to micronuclei is, at least in part, of nucleolar origin, for it contains both C23 and B23 proteins. It should be remembered that recent observations by Reipert et al. [1999] showed that in the pluripotent hematopoietic stem cell line, FDCP-Mix, nucleolar components contribute significantly to the content of the electron dense marginations. In the same article, it was also reported that a migration of DNA into the bulk of nucleolus takes place at an early stage of cell death and, therefore, it can be mistaken as condensed chromatin.

All in all, the antigens we investigated behaved in three different ways: (1) RNA pol I disappeared; (2) UBF associated with fibrogranular threaded bodies; and (3) fibrillarin, C23, and B23 remained detectable within material present amid micronuclei. In the future, it will be interesting to study whether or not other nucleolar constituents behave in one of these three ways, or if they follow a different behavior.

ACKNOWLEDGMENTS

This work was supported by a 1997 grant from the Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.) to A.M.M., Italian MURST 60% grants to Universita di Trieste and Urbino, and Cofinanziamento MURST 1998 to E.F. and Cofinanziamento MURST 1999 to E.F, A.M.M., and L.C.

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