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Biochemical and Morphological Characterization of the Nuclear Matrix From Apoptotic HL-60 Cells

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Abstract We have characterized the nuclear matrix-intermediate filament fraction from control and apoptotic HL-60 cells. Apoptosis was induced by exposure to the topoisomerase I inhibitor, camptothecin. By means of two-dimensional polyacrylamide gel electrophoresis, striking qualitative and quantitative differences were seen in the protein composition of the nuclear matrix–intermediate filament fraction obtained from apoptotic cells in comparison with controls. Western blotting analysis of apoptotic nuclear matrix proteins revealed degradation of some (topoisomerase IIα, SAF-A) but not other (SATB1 and nucleolin) components. Moreover, immunofluorescent staining for typical matrix antigens (NuMA protein, lamin B, SC-35) showed that in 35–40% of the structures prepared from apoptotic samples, marked changes in the subnuclear distribution of these proteins were present. Striking morphological differences between control and apoptotic samples were also detected at the ultrastructural level. These results demonstrate that both biochemical and morphological changes can be detected in the nuclear matrix prepared from apoptotic HL-60 cells. J. Cell. Biochem. 72:35–46, 1999.

Key words: apoptosis; nuclear matrix; scaffold/matrix-associated regions; protein degradation; immunocytochemistry

Apoptosis is a form of genetically encoded programmed cell death that plays an essential role in development, homeostasis, and defense in multicellular organisms [Bellamy et al., 1995; Jacobson et al., 1997; Leist and Nicotera, 1997]. Moreover, inappropriate apoptosis might be involved in the pathology of several human diseases, including cancer [Bellamy et al., 1995]. Apoptosis can be triggered by a variety of stimuli and is characterized by defined biochemical and morphological changes, mainly occurring at the nuclear level [Earnshaw, 1995; Kroemer et al., 1995; Hale et al., 1996; Vaux and Strasser, 1996]. In particular, the activation of multiple proteases is now recognized as an important event during apoptosis [Martin and Green, 1995]. These proteases include members of the interleukin-1β-converting enzyme/CED-3 family (caspases), serine proteases, calpains, and granzymes [Lazebnik et al., 1995; Patel et al., 1996]. Several proteins, of which many reside in the nucleus, have been recognized as targets of these proteases [Kaufmann, 1989; Casciola-Rosen et al., 1994; Oberhammer et al., 1994; Casiano et al., 1996; Waterhouse et al., 1996; Hsu and Yeh, 1996; Goehring et al., 1997; Gueth-Hallonet et al., 1997; see Martelli et al., 1997, for a recent review on this issue]. Also endonucleases, responsible for cleavage of genomic DNA into large (50–300 kbp, conceivably representing the loops in which the DNA is organized during the interphase) and small (140–160 bp, representing nucleosomal DNA) fragments, are activated during the apoptotic process [Hale et al., 1996; Vaux and Strasser, 1996; Collins et al., 1997]. Striking morphological changes at the nuclear level are a hallmark of apoptosis: Chromatin marginates toward the inner nuclear membrane forming cap-shaped, compact areas followed by complete condensa-

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tion of the chromatin and nuclear disruption into apoptotic bodies [Falcieri et al., 1994; Earnshaw, 1995; Stuppia et al., 1996].

Given these dramatic changes and considering also the fact that several of the proteolyzed proteins belong to the insoluble proteinaceous nuclear matrix fraction, which is thought to serve as a structural framework of the nucleus [Jack and Eggert, 1992; Berezney et al., 1995; Nickerson et al., 1995; Martelli et al., 1996], one would expect that the matrix showed evident changes during the apoptotic process. However, the findings of Arends et al. [1990] ruled out any differences both in the morphology and in the protein composition of nuclear matrix prepared from dexamethasone-treated thymocytes when compared to control. Subsequently, however, several articles have been published hinting at the fact that the nuclear matrix might be disassembled during the apoptotic process [Miller et al., 1993; Zweyer et al., 1995, 1997; Tinnemans et al., 1995; Weaver et al., 1996]. It is thought that chromatin is organized in loops with an average size of 50–100 kb interacting with the nuclear matrix through specialized AT-rich DNA regions called SARs (scaffold-associated regions) or MARs (matrix-associated regions) [Boulikas, 1995; Bode et al., 1996]. Several proteins of the nuclear matrix bind to S/MAR sequences in vitro and/or in vivo [Ludérus et al., 1992; Fackelmayer et al., 1994; Boulikas, 1995; Dickinson et al., 1995; Wang et al., 1995; Bode et al., 1996; Goehring and Fackelmayer, 1997]. It has been hypothesized that site-specific cleavage within S/MAR DNA sequences (conceivably favoured by proteolytic degradation of S/MAR binding proteins) could be linked to the higher-order DNA fragmentation which occurs during the initial stages of apoptosis [Stanulla et al., 1997; Goehring et al., 1997]. It is worth recalling that the nuclear matrix is composed of three well-distinct domains: a peripheral lamina, an internal fibrogranular network, and residual nucleoli [Berezeney et al., 1995]. So far, however, a detailed morphological and biochemical analysis of the changes occurring at the nuclear matrix level has never been presented. In this study, using nuclear matrix–intermediate filament fraction preparations [Fey et al., 1986; He et al., 1990; Nickerson et al., 1997], we show by means of immunofluorescent staining and ultrastructural analysis that dramatic morphological changes can indeed be detected at the nuclear matrix level in apoptotic HL-60 cells. We also demonstrate by two-dimensional gel electrophoretic separations and Western blotting analysis that the protein composition of the matrices from apoptotic samples differs significantly from control samples. Our results strengthen the contention that changes at the nuclear matrix level could be of fundamental importance to explain the mechanisms leading to the destruction of the nucleus during the apoptotic process.

MATERIALS AND METHODS

Cell Culture and Induction of Apoptosis

HL-60 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 for 3 h to 0.1 µg/ml of the DNA topoisomerase I inhibitor camptothecin (Sigma Chemical Co., St. Louis, MO), according to Del Bino et al. [1991].

Flow Cytometry

Cells were fixed with 70% ethanol for 30 min at 4°C. After two washes in phosphate-buffered saline (PBS), cells were stained with Coulter DNA-Prep reagent kit, according to the manufacturer's instructions. Analysis was carried out as previously described [Nicoletti et al., 1991] using an Epics XL flow cytometer (Coulter Immunology, Hialeah, CA).

Preparation of Nuclear Matrix-Intermediate Filament Fraction

This was done essentially as described by He et al. [1990]. Briefly, cells were first permeabilized in cytoskeletal buffer [10 mM Pipes, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 2 mM vanadyl ribonucleoside complex (VRC, Sigma), 1.2 mM phenylmethylsulfonyl fluoride, and 1 µg/ml of aprotinin and leupeptin]. They were then digested with 500 U/ml of RNase-free DNase I (Boehringer, Mannheim, Germany) for 60 min at 25°C in digestion buffer (10 mM Pipes, pH 6.8, 300 mM sucrose, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 2 mM VRC plus protease inhibitors as above). Subsequently, structures were first extracted with 0.25 M $(NH4)_2SO_4$ in 10 mM Pipes, pH 6.8, 300 mM sucrose, 3 mM

$MgCl_2$, 1 mM EGTA, 2 mM VRC plus protease inhibitors, and then with 2 M NaCl in the same buffer. The final pellets were used for all the following procedures.

Protein Recovery

Assays were performed as described by Bradford (1976).

Source of Antibodies

Monoclonal antibody to topoisomerase IIα (clone Ki-S1) was from Boehringer; monoclonal antibodies to lamin B and to the carboxy terminal domain of NuMA protein (clone 204-41) were from Oncogene Research Products, Cambridge, MA; a monoclonal antibody to the amino terminal domain (from 10 to 189) of NuMA was purchased from Transduction Laboratories, Lexington, KY; monoclonal antibody to splicing factor SC-35 [Spector et al., 1991] was obtained from Sigma; monoclonal antibody to protein C23/nucleolin (clone MS3) and human antiserum JO recognizing UBF protein [Ochs et al., 1994] were a kind gift of Dr. R.L. Ochs, Department of Molecular and Experimental Medicine, Scripps Research Institute, La Jolla, CA; polyclonal antiserum to SATB1 protein [Wang et al., 1995] was a kind gift of Dr. T. Kohwi-Shigematsu, Life Science Division, Lawrence Berkeley Laboratory, University of California, Berkeley, CA; polyclonal antiserum to SAF-A/hnRNP-U was as previously reported [Fackelmayer et al., 1994]. All of the aforementioned monoclonal antibodies are of IgG type except for the anti-NuMA from Transduction Laboratories, which is an IgM.

Sodium Dodecylsulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting Analysis

Nuclear matrix–intermediate filament structures (from 3×10^7 cells) were dissolved in electrophoresis sample buffer [Laemmli, 1970], and the proteins were separated on SDS-polyacrylamide gels that were then stained with Coomassie Brilliant Blue R-250. In some cases proteins were transferred to 0.2 µm nitrocellulose sheets for Western blotting analysis. After saturation with 4% bovine serum albumin (BSA) and 5% normal goat serum (NGS) in PBS for 1 h at 37°C, the sheets were incubated overnight at 4°C with the primary antibodies in PBS containing 4% BSA, 5% NGS. After washing in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 (TBST buffer), the sheets were incubated for 30 min at 25°C with peroxidase-conjugated secondary antibodies (anti-mouse IgG, anti-rabbit IgG, anti-human IgG, all from Sigma) diluted 1:3,000 in TBST buffer. After washing as above, antibody binding was revealed by enhanced chemiluminescence (Boehringer).

Two-Dimensional Gel Electrophoresis

Nuclear matrix–intermediate filament fraction protein (from 4×10^7 control or apoptotic cells) was resuspended in 400 µl of lysis buffer [O'Farrell, 1975] and incubated for 3 h at room temperature. Insoluble material was removed by centrifugation at 10,000*g* for 5 min, and the supernatant was layered on the first-dimension gel. Non-equilibrium pH gradient gel electrophoresis (NEPHGE) was carried out as reported by O'Farrell et al. [1977] in 9.2 M urea, 2% Nonidet P-40, 4% polyacrylamide, 2% ampholytes (Bio-Lyte pH 3–10, Bio-Rad Laboratories, Milan, Italy). First-dimension gels (10 cm long with a diameter of 3 mm) were run for 16 h at 270 V (constant). Second-dimension gels were 8% polyacrylamide-0.1% SDS slabs. Gels were stained with Coomassie Blue R-250. The pH gradient was calibrated using carbamylated glyceraldehyde-3-phosphate dehydrogenase standards (Pharmacia Biotech, Uppsala, Sweden).

Immunofluorescent Staining

The procedure was carried out essentially as described by Neri et al. [1997]. Nuclear matrix structures in PBS 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 PBS for 30 min at room temperature. After several washes with PBS, nonspecific binding of antibodies was blocked by a 30 min incubation at 37°C with PBS, 2% BSA, 5% NGS. Slides were then incubated for 3 h at 37°C with the appropriate primary antibody diluted in PBS, 2% BSA. Slides were then washed three times in PBS and reacted with fluorescein isothiocyanate–conjugated anti-mouse IgG or IgM, diluted 1:100 in PBS, 2% BSA, 5% NGS for 1 h at 37°C. Samples were subsequently washed three times in PBS, stained for DNA with 0.5 µg/ml DAPI (4′-6-diamidino-2-phenylindole) 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 (BDH, Poole, England). Slides were observed and photographed using a Zeiss Axiophot epifluorescence microscope.

Transmission Electron Microscopy (TEM) Analysis

Structures were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 35 min. They were embedded in Araldite and observed with a Jeol 100S electron microscope.

RESULTS

To study the structure and composition of the nuclear matrix from apoptotic cells, we have used HL-60 cells, which can be induced to undergo apoptotic cell death by exposure to camptothecin, an inhibitor of topoisomerase I [Del Bino et al., 1991]. After 3 h of exposure to camptothecin, approximately 70% of the cells exhibited apoptotic features, both morphologically and biochemically, as shown by flow cytometric analysis (Fig. 1a). In contrast, <1% of control cells were apoptotic (Fig. 1b).

Apoptotic as well as non-apoptotic control cells were investigated for their nuclear matrix according to the well-characterized protocol of He et al. [1990]. With this protocol, cells are successively extracted with non-ionic detergents, 0.25 M $(NH_4)_2SO_4$, and 2 M NaCl, finally resulting in a so-called nuclear matrix–intermediate filament fraction.

SDS-PAGE analysis of the polypeptides present in this fraction revealed striking quantitative differences between control and apoptotic cells (Fig. 2A, compare lane 1 with lane 3 and lane 2 with lane 4). No major differences were seen between samples extracted with 0.25 M $(NH4)_2SO_4$ only and those also treated with 2 M NaCl (see Fig. 2A, compare lane 1 with lane 2 and lane 3 with lane 4). This is consistent with the results of He et al. [1990]. Protein assay revealed that the nuclear matrix–intermediate filament fraction from control cells retained 13.4 ± 2.4% (means ± SD, n = 3) of cell protein, whereas samples from apoptotic cells retained only 7.5 ± 1.8%.

Since the polypeptide composition of the nuclear matrix is exceedingly complicated [Berezney et al., 1995], we analyzed by two-dimensional gel electrophoresis the proteins of the nuclear matrix–intermediate fraction prepared from control and apoptotic samples (Fig. 2B,C). It should be emphasized that in this case we

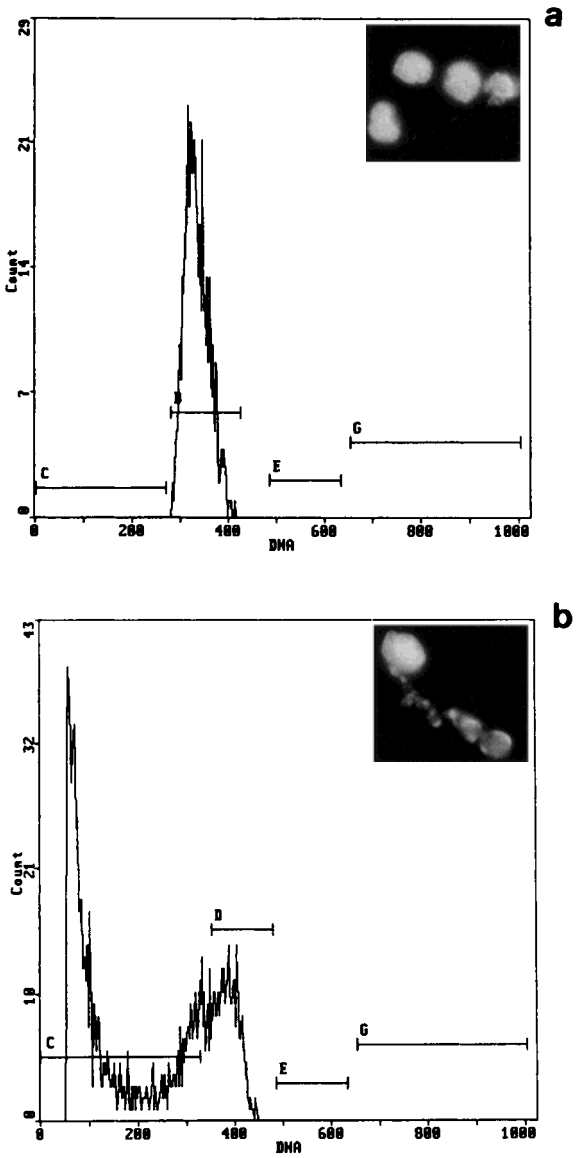

Fig. 1. Flow cytometric analysis of control (**a**) and apoptotic (**b**) HL-60 cells. Since control cells were just released from the aphidicolin block (see Materials and Methods), they are all at the G_1/S border. C: apoptotic cells; D: G_1-phase cells; E: S-phase cells; G: G_2/M-phase cells. The insets in a and b show the typical aspect of normal (a) and apoptotic nuclei (b) as revealed by DAPI staining.

studied the polypeptides in samples extracted with 0.25 M $(NH4)_2SO_4$ only. It is evident that both quantitative and qualitative differences were seen between apoptotic and control cells, confirming the result of the one-dimensional analysis shown in Figure 2A. As an example of striking quantitative differences we have indicated two spots (A and B, arrows). Because of its characteristic migration features in this electrophoretic system, spot B corresponds to lamin

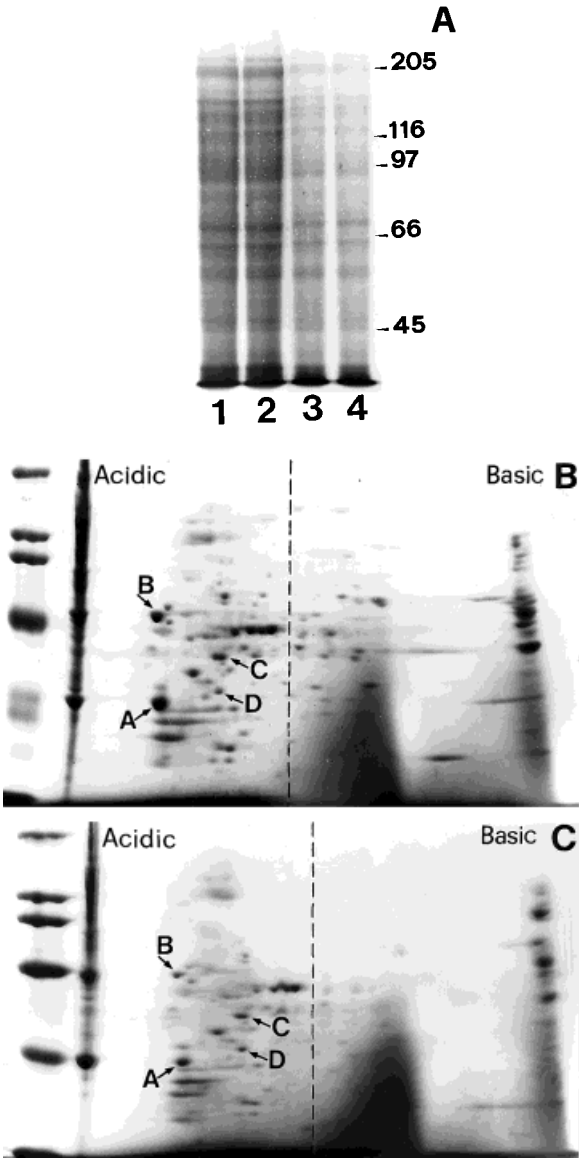

Fig. 2. SDS-PAGE (**A**) and two-dimensional gel electrophoresis (**B** and **C**) analysis of proteins recovered in the nuclear matrix-intermediate filament fraction from control and apoptotic HL-60 cells (exposed for 3 h to camptothecin). A: **Lanes 1 and 2** are control cells; **lanes 3 and 4** are apoptotic cells. In each lane we loaded protein recovered from 3×10^7 cells. In lanes 1 and 3, samples have been extracted with 0.25 M $(NH4)_2SO_4$, whereas in lanes 2 and 4 they have also been treated with 2 M NaCl (see Materials and Methods). A 8% polyacrylamide gel was employed. B and C: In B we separated protein from control samples, whereas in C we ran protein from apoptotic samples. Migration in the first dimension was from left to right. The dashed lined indicates pH 7.0. Molecular weight standards (on the left) are, from top to bottom, 205, 116, 97.5, 66, 45, and 29 kDa.

B [Martelli et al., 1995; Berezney et al., 1995]. Spot A (Mr about 45 kDa, with an electrophoretic mobility in the first dimension equal to lamin B) likely corresponds to spot 13 of the classification of Berezney et al. [1995]. However, we have also indicated two other spots (C and D, arrows) which do not show evident differences between control and apoptotic samples.

We next performed Western blotting analysis on control and apoptotic samples of nuclear matrix–intermediate filament fraction using antibodies to some well-established matrix components. It is worth emphasizing that four out of five polypeptides we have investigated show S/MAR-binding activity (i.e., SAF-A, topoisomerase IIα, nucleolin, and SATB1; see Boulikas [1995] for a review on this issue), while UBF is an accessory factor to RNA polymerase I [Roussel et al., 1993].

As presented in Figure 3, in apoptotic samples we observed a slight reduction of SAF-A (in this case some degradation products were seen) and a dramatic decrease of UBF. Topoisomerase II α was almost completely degraded to multiple fragments with a Mr <170 kDa in apoptotic samples, in agreement with Casiano et al. [1996]. In contrast, we did not see changes involving nucleolin and SATB1.

We then investigated by immunofluorescent staining whether changes in subnuclear distribution of matrix proteins could be detected in apoptotic samples. The results are presented in Figure 4. Also in this case we extracted samples with 0.25 M $(NH4)_2SO_4$ only. Staining of the structures with 4′-6-diamidino-2-phenylindole (DAPI) revealed complete removal of DNA in both control and apoptotic samples (data not shown and Nickerson et al. [1997]). As a general observation it should be stressed that in about 35–40% of nuclear matrices prepared from apoptotic samples we detected a dramatic reduction of their dimension, with a diameter about 30–35% smaller than in control samples. Indeed, the diameter of control matrices ranged between 7.5 and 8.0 μm, whereas in apoptotic samples it comprised between 4.5 and 5.5 μm. To us this suggested that at least a partial collapse of an internal nuclear structure could have taken place.

We employed two different antibodies to NuMA protein, one recognizing the amino terminal and the other directed to the carboxy terminal of the polypeptide. In control samples the antibody to the amino terminal stained the nuclear matrix in quite a diffuse manner; however, it also decorated several spots of different size located both in the center and at the periphery of the structures (Fig. 4a). In apoptotic matrices some spots of different size were still present, but the diffuse staining was almost

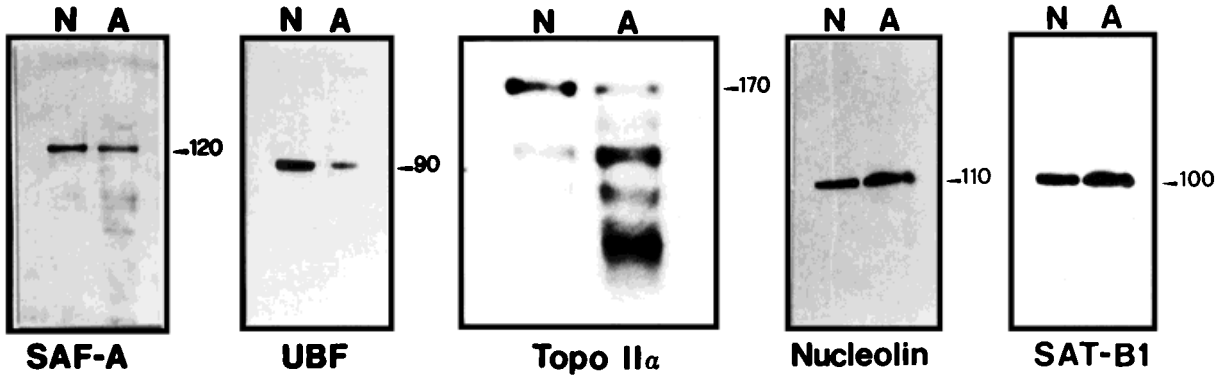

Fig. 3. Western blotting analysis for SAF-A, UBF, topoisomerase IIα, nucleolin, and SATB1 in normal (N) and apoptotic (A) nuclear matrix-intermediate filament fraction. To each lane we blotted protein recovered from 1.5×10^7 cells. The molecular weight of the native protein is indicated to the right of each panel.

abolished (Fig. 4b). The antibody to the carboxy terminal of NuMA immunostained a fibrogranular network. Negative areas were also present in between the network (Fig. 4c). In apoptotic samples we detected numerous spots of different shape, mainly localized to the center of the structures (Fig. 4d). Antibody to lamin B decorated the periphery of the matrices forming a ring (Fig. 4e). Nevertheless, some intranuclear staining in the form of filamentous structures was also present, in agreement with the findings of Moir et al. [1994]. In apoptotic samples the peripheral ring was almost absent, and the intranuclear staining was very fine and dispersed all over the structures except for negative areas possibly corresponding to nucleolar remnants (Fig. 4f). No filamentous structures were observed. SC-35 antibody stained numerous spots, of different size, located both in the center and at the periphery of the structures (Fig. 4g). In apoptotic matrices, besides some residual spots, the antibody immunodecorated large and extremely bright masses (Fig. 4h).

Transmission electron microscope (TEM) analysis of the nuclear matrix–intermediate filament fraction prepared with 0.25 M $(NH_4)_2SO_4$ from control cells showed a typical morphology consisting of a peripheral lamina with remnants of the cytoskeleton, an inner fibrogranular network, and residual nucleoli (Fig. 5A). Given the fact that HL-60 cells, like many hemopoietic cell lines, contain few cytoskeletal proteins [Wang and Traub, 1991], cytoskeletal remnants were hard to detect, at variance with the results of Nickerson et al. [1997] who employed an epithelial cell line (Caski cells) containing a much more developed cytoskeleton. In contrast, when the fraction was obtained from cells exposed for 3 h to camptothecin, about 35–40% of the structures lacked residual nucleoli as well as the inner network (Fig. 5B). They were homogeneously electron dense in their interior, displayed a highly irregular shape, and in some segments of the periphery exhibited a dark edge, possibly related to the maintenance of some components of the nuclear lamina. Often, some electron-transparent vesicles were seen inside the structures. It should also be stressed that these structures were smaller than normal matrices, with a diameter about 45–50% smaller than control matrices. If the extraction was also carried out with 2 M NaCl, 35–40% of the structures were about two- or threefold larger than control matrices (Fig. 5C), and they were also irregular in shape. At the periphery, they displayed a very evident ring, whereas while a medium electron-density amorphous material was present in the interior. Since Arends et al. [1990] exposed isolated nuclei to the cross-linking agent sodium tetrathionate (NaTT [Stuurman et al., 1992]) prior to preparation of the nuclear matrix from apoptotic thymocytes by digestion with DNase I and extraction with high-ionic-strength buffer, we sought to determine whether this treatment might have affected their results, because it is well known that this chemical causes a stabilization of the internal matrix and residual nucleoli that could be artifactual [Martelli et al., 1996]. In fact, if the extraction was also carried out with 2 M NaCl we did not see the empty and large structures presented in Figure 5C. In contrast, several different structures were identified: 1) homogeneously dense matrices, like those presented in Figure 5B (data not presented); 2) isolated residual nucleoli (Fig.

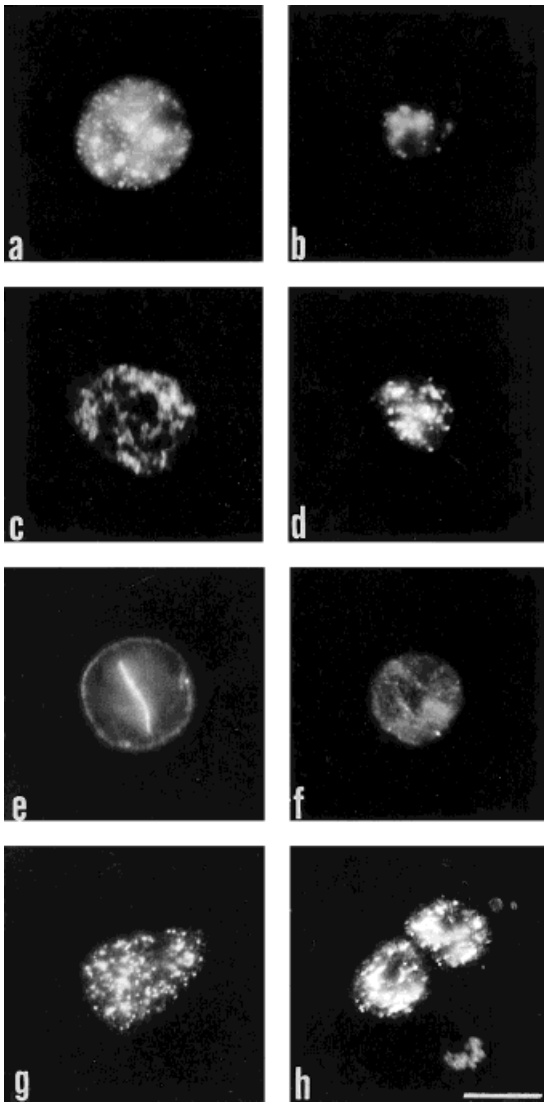

Fig. 4. Immunofluorescent staining of control (**a,c,e,g**) and apoptotic (**b,d,f,h**) nuclear matrices stained for amino terminal domain (a,b) and carboxy terminal domain (c,d) of NuMA protein; lamin B (e,f); SC-35 (g,h). Scale bar = 5 µm.

5D); 3) matrices with their fibrogranular network in the process of being degraded (Fig. 5E). Nucleolar remnants in apoptotic samples sometimes showed a large inner clear area (Fig. 5D and E), as described for intact apoptotic HL-60 cells [Zweyer et al., 1995]. In this case, residual nucleoli presented also very electron-dense lenticular structures that were localized within the peripheral ring surrounding the internal electron-transparent area (Fig. 5D,E). Frequently, we also saw isolated nucleolar remnants still displaying a segregation of their components (Fig. 5F), as reported for intact apoptotic HL-60 cells [Zweyer et al., 1997].

DISCUSSION

Although the morphological changes occurring at the nuclear level during apoptosis have long been recognized, the molecular mechanisms underlying them have escaped clarification for many years. The finding that several nuclear matrix proteins are proteolyzed during the apoptotic process together with the demonstration that the large chromatin fragments correspond to DNA loops has drawn attention to the fact that the nuclear matrix could be disassembled during apoptosis. However, in the past, Arends et al. [1990] ruled out biochemical and ultrastructural changes in the nuclear matrix prepared from apoptotic thymocytes. Nevertheless, indirect evidence suggesting a likely involvement of the nuclear matrix during apoptosis came from results obtained by means of immunofluorescence and/or immunoelectron microscopy techniques and showing unstainability of apoptotic nuclei with antibodies directed to nuclear matrix components as well as the presence in nuclei of apoptotic cells of granular and threaded bodies containing several nuclear matrix polypeptides [Miller et al., 1993; Zweyer et al., 1995; Weaver et al., 1996; Biggioggera 1997a,b; Zweyer et al., 1997; Lafarga et al., 1997]. However, firm evidence suggesting an involvement of the proteinaceous nuclear matrix during the apoptotic process was still missing. We reasoned that the nuclear matrix fraction of apoptotic samples could be very fragile. Therefore, to isolate it, we chose to employ the method devised by Penman and coworkers [e.g., He et al., 1990], which is by far the gentlest devised up to now [Martelli et al., 1996]. In this method, whole cells are permeabilized with a non-ionic detergent to remove membranes and soluble proteins, and then the samples are treated with nucleases and salt solutions to remove chromatin. At the end, the so-called nuclear matrix–intermediate filament fraction is obtained. It is worth emphasizing that with this technique we avoided the use of low-ionic-strength buffers to isolate nuclei, i.e., conditions which are known to be a potential source of artifacts [Nickerson et al., 1995; Martelli et al., 1996]. A major issue is that we have demonstrated that the protein composition of the nuclear matrix–intermediate fraction from apoptotic samples is markedly different, both quantitatively and qualitatively, from controls. This is at variance with Arends et al. [1990].

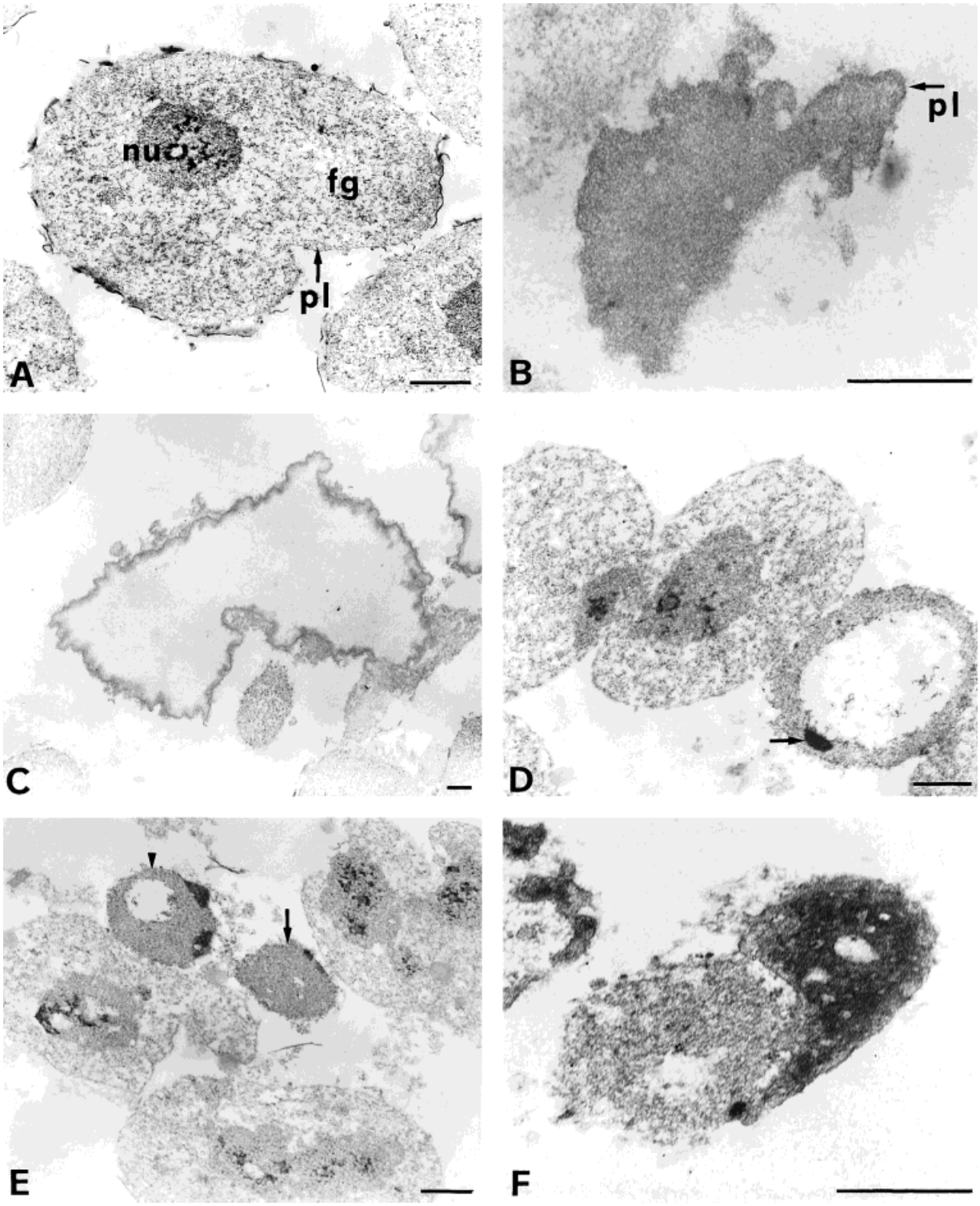

Fig. 5. Transmission electron microscope analysis of nuclear matrix-intermediate filament fraction from control and apoptotic HL-60 cells. **A:** Micrograph of nuclear-matrix intermediate filament fraction prepared from control cells. nu, residual nucleoli; fg, fibrogranular network; pl, peripheral lamina. **B:** These dense, homogeneous structures could be observed when samples from apoptotic cells have been extracted with 0.25 M $(NH_4)_2SO_4$ only. pl, peripheral lamina. **C:** These extremely large structures were seen in apoptotic samples extracted also with 2 M NaCl. **D–F:** The apoptotic samples were treated with 2 mM NaTT prior to DNase I digestion and salt extraction (both 0.25 M $(NH_4)_2SO_4$ and 2 M NaCl). In D an isolated nucleolar remnant presenting an inner electrontransparent area lies close to an apparently normal matrix. The nucleolar remnant shows a lenticular electron-dense granule near its periphery (arrow). In E a partially degraded matrix (arrowhead) containing a nucleolar remnant with the inner electron transparent area and the dark lenticular peripheral granules. An isolated nucleolar remnant is also indicated (arrow). F: An isolated nucleolar remnant still exhibiting segregation of its components. Scale bars = 1 μm.

The difference in the protein composition was assessed both by Coomassie Blue staining of two-dimensional gels and by Western blotting analysis using antibodies to well-characterized matrix components. In this connection, it should be recalled that very recently Gerner et al. [1998] described alteration of nuclear matrix protein composition (assessed by two-dimensional gels) during apoptosis of rat embryo cells. However, they detected in samples from apoptotic cells various proteins that were not present in controls.

Our results also indicated that there are matrix polypeptides which are unaffected by the apoptotic process (for example SATB1 and nucleolin) in agreement with the recent findings of Casiano et al. [1996]. It is worth emphasizing that two of the matrix polypeptides that underwent proteolysis in our system (SAF-A and topoisomerase IIα) have S/MAR-binding capacity, but two other proteins with the same binding characteristic (SATB1 and nucleolin) apparently were not proteolyzed, since we recovered the same amount in control and apoptotic matrices. SAF-A, SATB1, and topoisomerase IIα are components of the matrix inner network, whereas nucleolin is mainly localized to the nucleolus [Martelli et al., 1996]. We have recently demonstrated that the 240-kDa NuMA protein (also possessing S/MAR-binding features) is proteolyzed in HL-60 cells exposed to camptothecin [Zweyer et al., 1997]. Interestingly, SATB1 is proteolyzed in apoptotic thymocytes [Martelli et al., 1997]. This might indicate that, depending on the effector and/or the cell line, different S/MAR-binding proteins are proteolytically degraded to facilitate DNA cleavage, in agreement with the suggestion by Kizaki and Onishi [1997]. We feel that such a hypothesis should be tested in a rigorous manner, and experiments are underway in our laboratory. It is worth noting that for some proteins we recovered in the matrix fraction the proteolyzed fragments (SAF-A and topoisomerase IIα), whereas for UBF we did not see them, although Casiano et al. [1996] have described the presence of multiple fragments, comprising between 24 and 55 kDa. It might be that these fragments are extracted by detergent and/or salt buffers. However, it might also be that UBF is not proteolyzed but simply becomes more extractable as a result of changes in the nucleolar structure.

Also immunofluorescent staining to some matrix components suggested that at least a partial collapse of nuclear matrix is likely to take place in apoptotic nuclei. The fact that by this approach we detected impressive changes in the subnuclear distribution of NuMA protein appears to be particularly noteworthy, because this protein displays structural features such as a globular head, tail domains, and a central α-helical rod domain similar to those of the member of the family of intermediate filament proteins [He et al., 1995]. Therefore, NuMA might be a true structural component of a nuclear framework, given also the fact that it is a constituent of the matrix "core filaments" [Nickerson et al., 1995]. It is important to note that very recently Sodja et al. [1997], studying apoptotic lymphocytes, have detected changes in the subnuclear distribution of NuMA protein very similar to ours. Nevertheless, their results were obtained in intact cells, whereas ours have been seen in isolated nuclear matrices. This indicates that the procedure for isolating the matrix did not alter the distribution of NuMA in apoptotic samples. We also detected changes in the distribution of lamin B, not only at the nuclear periphery but also within the nucleus. Because also lamin proteins belong to the family of intermediate filaments and could play a structural role inside the nucleus and not only at its periphery [Hozak et al., 1995], a redistribution of lamin B might be another clue to the collapse of an internal matrix. The changes in the distribution of lamin B conceivably reflect also degradation of this polypeptide. Indeed, in two-dimensional gels we have observed a decrease in the amount of lamin B, while by Western blotting analysis we detected degradation of this protein (A.M. Martelli and R. Bortul, unpublished experiments) in agreement with several other investigators [e.g., Oberhammer et al., 1994; Weaver et al., 1996]. The SC-35 antibody recognizes an essential, salt-resistant splicing component [Spector et al., 1991] that mainly localizes to a peculiar nuclear domain referred to as "speckles." Our results indicate that this domain is also affected during the apoptotic process in HL-60 cells. Finally, transmission electron microscope observations demonstrated the presence, in apoptotic samples, of matrix structures markedly different from controls. The homogeneously electron-dense structures seen in samples extracted with 0.25 M $(NH4)_2SO_4$ only reminded us of the smaller nuclear matrices detected in apoptotic samples by immunofluorescent staining. Indeed,

with both techniques we estimated that about 35–40% of the structures presented altered morphological features. At present, we have no explanation for the extremely large structures detected in samples extracted also with 2 M NaCl; perhaps they are derived from aggregation of several apoptotic matrices. Stabilization with NaTT was only partially effective in that after extraction with 2 M NaCl we did not observe the large structures but instead we recognized the homogeneously electron-dense structures that in the absence of a stabilization were present when the extraction was carried out with 0.25 M $(NH4)_2SO_4$ only. However, the use of this chemical cross-linking agent allowed us to preserve in the final matrix fraction some morphological features that are usually seen in nucleoli of intact apoptotic HL-60 cells, such as the presence of a large clear inner area and segregation of nucleolar components [Zweyer et al., 1995, 1997]. Under these conditions we also observed still-recognizable nucleolar remnants devoid of the surrounding inner matrix network. In our opinion this observation demonstrates that the nucleolus (or at least some of its components) may be stabilized by NaTT even in the most advanced phases of the apoptotic process when the inner network is completely degraded and is in agreement with previous findings hinting at the nucleolus as the nuclear domain that is most resistant to apoptosis, even if also some of its components, such as UBF, are cleaved [Falcieri et al., 1994; Zweyer et al., 1995; Casiano et al., 1996]. Although morphological changes at the light and electron microscope level were detected in only 30–35% of the final structures, it should be reminded that not all of the cells underwent apoptosis at the same time (70% were apoptotic after 3 h treatment, as judged by flow cytometry), and the process does not take place in a synchronous manner. It is conceivable that it is quite difficult to recognize the earliest morphological changes occurring in the nuclear matrix during apoptosis (for example, when the nucleus only displays a margination of condensed chromatin), and probably, when the apoptotic process reaches its latest stages (i.e., the presence of micronuclei) no matrix fraction at all can be isolated.

The discrepancy between our findings and those by Arends et al. [1990] might be due to at least two reasons. First, they used a different cell system and inducer (i.e., rat thymocytes treated with steroids). Second, to prepare the nuclear matrix we have employed probably the most gentle procedure available, whereas they used a harsher technique. It might be that the structures displaying an altered morphology would not have been detectable if we had employed a different method to prepare the matrix, which would have resulted in a destruction of the more fragile structures. It will be interesting in the future to test the aforementioned hypotheses to clarify the reason(s) responsible for the different results. Taken together, our results strengthen the concept that destruction of the nuclear matrix is one of the key events leading to chromatin collapse and nuclear breakdown in apoptotic cells [Martelli et al., 1997]. Future investigation in this field should provide additional information on the molecular mechanisms underlying nuclear destruction.

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