available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Dynamic relocation of nuclear proteins during the execution phase of apoptosis

Anna Ivana Scovassi [a,*], *Maria Grazia Bottone* [a,b], *Marco Biggiogera* [a,b], *Carlo Pellicciari* [b]

[a] *Istituto di Genetica Molecolare CNR, Via Abbiategrasso 207, I-27100 Pavia, Italy*
[b] *Department of Animal Biology, University of Pavia, Piazza Botta 10, I-27100 Pavia, Italy*

ARTICLE INFO

Article history:
Received 14 May 2008
Accepted 10 June 2008

Keywords:
Apoptosis
Fluorescence and electron microscopy
HERDS
Immunocytochemistry
Nuclear proteins
RNPs

ABSTRACT

In the apoptotic program of controlled cell dismantling, the most characteristic nuclear changes involve chromatin, which condenses and often collapses against the nuclear envelope in the form of crescents. A severe reorganization also occurs in ribonucleoprotein (RNP)-containing structures which are involved in the synthesis and processing of transcripts: already during early apoptosis, the nucleoplasmic RNPs (namely, perichromatin fibrils, perichromatin granules, and interchromatin granules) coalesce in the interchromatin space where they associate with segregated nucleolar components, to ectopically form fibro-granular heterogeneous clusters. This was found to occur in cell systems in vivo and in cultured cell lines, after different apoptogenic stimuli. These RNP aggregates we have called heterogeneous ectopic RNP-derived structures (HERDS) move from the nucleus to the cytoplasm, and may be found in apoptotic bodies, in late apoptosis. Immunolabeling experiments demonstrated that several other proteins which are normally located inside the nucleus also move into the cytoplasm, during apoptosis, independently from HERDS. Apoptotic cells have been suggested to be a powerful source of nuclear auto-antigens, which are produced by the partial proteolytic or nucleolytic cleavage of a wide variety of nuclear substrates. In the presence of defective phagocytosis (or when massive apoptosis overwhelms the clearance capability of the tissue scavenger cells), the disposal of apoptotic cells becomes insufficient and unphagocytosed late apoptotic cells may accumulate in the tissue where they may be engulfed by antigen-presenting cells (such as dendritic cells); an autoimmune response may thus be elicited, by which apoptosis-derived auto-antigens are recognized and presented to the immune system.

1. Introduction

Apoptosis is the regulated form of physiological cell death, which occurs during the embryonic development and is responsible for the maintenance of tissue homeostasis in adult tissues [1,2].

Apoptotic cell death takes place through a largely stereotypic series of cellular events which allow the safe and progressive dismantling of the inner cell structures: both the nucleus and cytoplasm undergo impressive restructuring, with massive chromatin condensation and nuclear fragmentation, structural and functional reorganization of organelles,

* *Corresponding author*. Tel.: +39 0382 546334; fax: +39 0382 422286.
E-mail address: scovassi@igm.cnr.it (A.I. Scovassi).
0006-2952/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2008.06.005

and the release of apoptotic bodies which are finally engulfed by neighbouring phagocytic cells [3]. The exposure at the cell surface of the apoptotic cells and bodies of "eat-me" molecular signals promotes their recognition and rapid removal by phagocytes [4–6].

The massive reorganization of the nucleus and the organelle structure in apoptotic cells has the obvious consequence of progressively destroying the whole cell biosynthetic machinery, while the integrity of the plasma membrane is maintained, thus preventing the harmful leakage of cellular contents into the extracellular space.

In recent years, the main molecular mechanisms at the basis of the induction and progress of apoptosis have been elucidated, and it is now widely accepted that the morphological apoptotic changes can be, either directly or indirectly, related to the concerted activation of proteolytic enzymes called caspases (reviewed in Refs. [7,8]). These proteases are constitutively present in the cell in an inactivated form, and are activated through the proteolytic cleavage taking place in a cascade manner: the so-called "initiator" caspases then act on "effector" caspases which in turn cleave a wide variety of cytoplasmic and nuclear substrates. This proteolytic degradation can either directly disrupt structural components or inactivate regulatory proteins. As a consequence of these cleavage events, the activation of endogenous nucleases promotes the degradation of DNA, first into high-molecular-weight pieces (of about 300–50 kb) and finally into oligonucleosome-long fragments which are recognized as the typical ladder in conventional agarose gel electrophoresis (reviewed in Ref. [9] and, more recently, in Ref. [10]).

During the execution phase of apoptosis, the cell undergoes shrinkage and fragmentation into apoptotic bodies: it is now accepted that these dramatic changes in cell organization depend on a series of proteolytic and nucleolytic events taking place almost simultaneously in both the cytoplasm and nucleus, and that this may occur through the dynamic redistribution of protein factors which move from their cytosolic or organelle stores to other cytoplasmic and/or nuclear locations [11,12].

No doubt, the nucleus is the site where the most prominent changes take place during apoptosis. The nucleus is an extremely complex organelle, where at least two largely interacting compartments may be recognized by microscopical techniques [13–16]: chromatin and the interchromatin space where ribonucleoprotein (RNP)-containing structures are present. By the rather simple ultrastructural technique based on a treatment with EDTA [17–20], condensed chromatin may be quite efficiently bleached whereas the interchromatin space becomes more contrasted: here, besides the nucleolus (which is the most prominent RNP-based intranuclear body), some other RNP-containing structures can be morphologically recognized which proved to be part of the transcription and splicing machinery. At electron microscopy, these structures have been described as perichromatin fibrils (PF), perichromatin granules (PG), and interchromatin granules (IG) (reviewed in Ref. [14]). PF are the sites where hnRNA transcription and co-transcriptional splicing occur [21–23]; in the IG, snRNP and non-snRNP splicing factors are stored [21] and possibly spliceosome assembly takes place [24]; PG are involved in the storage of mRNA and its transport from the nucleus to the cytoplasm [21]. All these components have a specific intranuclear location, which is a necessary prerequisite for the maturation of nuclear RNAs (reviewed in Ref. [25]): PF and PG reside at the periphery of condensed chromatin whereas IG locates in the so-called interchromatin space.

Actually, during apoptosis, the massive chromatin restructuring is paralleled by a severe reorganization of the nucleolus and the non-nucleolar RNPs too [26,27]. In the present paper, we used immunocytochemical techniques at light and electron microscopy to investigate the relocation of nuclear RNPs and some other nuclear proteins during the execution phase of apoptosis, in the attempt to describe the dynamics of the nuclear disassembly and to elucidate the fate of these nuclear components in late apoptosis.

2. Materials and methods

2.1. Cells and apoptogenic treatments

Thymocytes have been used as an example of cell population in which apoptosis spontaneously occurs: they were collected from 3-week old rats by mechanical disaggregation of the thymuses in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 100 units each of streptomycin and penicillin (Celbio, Milano, Italy). To induce massive apoptosis, thymocytes were also resuspended in complete medium containing 10 μM etoposide for 1 h, and then allowed to grow for additional 4 h in drug-free medium (as reported in Ref. [28]).

Apoptosis was also induced by different treatments (see Table 1) in the mammalian cell lines listed in Table 2; all these adherent cells were grown in the appropriate medium supplemented with FBS, glutamine and antibiotics as above,

Table 1 – Apoptogenic treatments

Treatment	Drug concentration	Time length	Ref.
Etoposide	10–100 μM	1–24 h	[28,29]
Actinomycin D	0.01–1 μg/ml	15 h	[30]
Hypertonic stress	0.246 M NaCl	1–10 days	[31]
Cisplatin	4–40 μM	20, 24, 72 h	[32,33]
Photosensitization with rose Bengal acetate	10^{-6} M for 1 h followed by irradiation with green light	24–72 h post-irradiation	[34]
Taxol	40 nM	6, 24, 48 h	[35]

All reagents were from Sigma–Aldrich (Milano, Italy), with the exception of rose Bengal acetate which was purchased from Molecular Probes (Invitrogen, San Giuliano Milanese, Italy).

Table 2 – Cell types

Cell types	Origin	Species	Ref.
Thymocytes	Thymus	Rat and mouse	[28]
Cells of external granular layer	Cerebellum	Rat	[32]
HeLa	Uterin cervix carcinoma	Human	[30,34]
C6	Glioma	Rat	[36]
B50	Neuroblastoma	Rat	[33]
SW613	Colon carcinoma	Human	[35]
EUE	Embryonic epithelium	Human	[31]

The cell culture conditions used in the present investigation have been described in the reported references.

and seeded either in 25 cm^2 plastic flasks or on glass coverslips in Petri dishes; the cells were planted at least 24 h before applying the apoptogenic treatments when they were in exponential growth.

2.2. Immunocytochemistry at fluorescence microscopy

The cells were grown on glass coverslips and processed for the single or dual immunodetection of the antigens listed in Table 3, which were visualized by the appropriate fluorochrome-labeled secondary antibodies (Molecular Probes, Invitrogen, San Giuliano Milanese, Italy). The immunolabeled cell samples were counterstained for DNA with 0.1 μg/ml Hoechst 33258 (Sigma–Aldrich, Milano, Italy) in PBS for 15 min, and finally mounted with Mowiol (Calbiochem, La Jolla, CA, USA).

For observation and micrography, an Olympus BX51 microscope (Olympus Italia S.r.l., Segrate, Italy) equipped with a 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 Hoechst; 450–480 nm excf, 500 nm dm and 515 nm bf for Alexa 488; and 540 nm excf, 580 nm dm, and 620 nm bf for Alexa 594. The images were recorded with an Olympus Camedia 5050 digital camera or an Olympus Magnifire digital camera system and stored for processing on a PC by the Olympus software.

For confocal laser scanning microscopy, we used a Leica TCS-SP system mounted on a Leica DMIRBE inverted microscope (Leica Microsystems S.p.A., Milano, Italy); for fluorescence excitation, an Ar/UV laser at 364 nm was used for Hoechst 33258, an Ar/Vis laser at 488 nm for FITC and a He/Ne laser at 543 nm for Alexa 594. Spaced (0.5 μm) optical sections were recorded using a 63× oil immersion objective. Images were collected in the 1024 × 1024 pixel format, stored on a magnetic mass memory and processed by the Leica confocal software. 0.5 μm spaced optical sections were recorded using a 63× oil immersion objective.

2.3. Electron microscopy: morphology and immunocytochemistry

For the morphological analysis, the cells were harvested by mild trypsinization (0.25% trypsin in PBS containing 0.05% EDTA) and immediately fixed with 2% glutaraldehyde (1 h at 4 °C) in the culture medium and post-fixed in 1% OsO_4 for 1 h at room temperature. The cell pellets were embedded in 2% agar in water, thoroughly rinsed with PBS (pH 7.2) and dehydrated in ethanol. Finally, the cells were embedded in LR White resin (Società Italiana Chimici, Roma, Italy) and the ultrathin sections were routinely stained with uranyl acetate and lead citrate.

For the immunocytochemical analysis, the cells were harvested by mild trypsinization as above, and then fixed in suspension with 2% *p*-formaldehyde containing 0.2% glutaraldehyde in D-MEM medium for 1 h at 4 °C. The samples were centrifuged and embedded in 2% agarose, to be

Table 3 – Primary antibodies used in immunolabeling experiments

Antibody recognizing	Type	Dilution	Ref.
hnRNP core proteins	Chicken polyclonal	1:3000	[37]
Sm antigen (U_1, U_2, $U_{4/6}$, U_5 snRNP)	Human autoimmune serum	1:200	[37]
U2AF65	Rabbit polyclonal	1:100	[38]
CFI_m	Rabbit polyclonal	1:500	[39]
CStF	Chicken polyclonal	1:500	[40]
SC-35	Mouse monoclonal	1:100	[30]
P_0, P_1, P_2	Mouse monoclonal	1:100	[37]
Fibrillarin	Human autoimmune serum	1:800	[41]
Ki-67	Mouse monoclonal	1:20	[41]
Phosphorylated c-Myc	Rabbit polyclonal	1:100	[41]
p89 (proteolytic fragment of PARP-1)	Rabbit polyclonal	1:100	[29]
CENP-B	Human autoimmune serum	1:200	[42]

The cells were incubated for 1–2 h at room temperature with the primary antibody; the commercial source, concentrations and the incubation times for the primary and secondary antibodies have been reported in the cited references. Human autoimmune sera were kindly provided by Dr. Claudia Alpini (IRCCS San Matteo, Pavia, Italy).

dehydrated in ethanol and finally embedded in LRWhite resin. Sixty to seventy nanometers thick sections were cut and mounted on nickel grids. At the end of all the immunocytochemical procedures, the sections were stained with Bernhard's regressive EDTA method [17], to allow detection of the nuclear structures containing RNPs. The ultrathin sections processed for either morphology or immunocytochemistry were examined and photographed under a Zeiss TEM 900 operating at 80 kV (Carl Zeiss S.p.A., Arese, Italy).

3. Results

As it can be easily observed at light and electron microscopy, chromatin condensation is known to start along the nuclear envelope with a margination which then extends to form thicker crescent-shaped aggregates; in late apoptosis, karyorrhexis occurs and the nucleus is fragmented into round-shaped dense bodies. Based on this morphological evidence, and for sake of simplicity, we operationally defined three phases of the apoptotic process, i.e. early (incipient condensa-

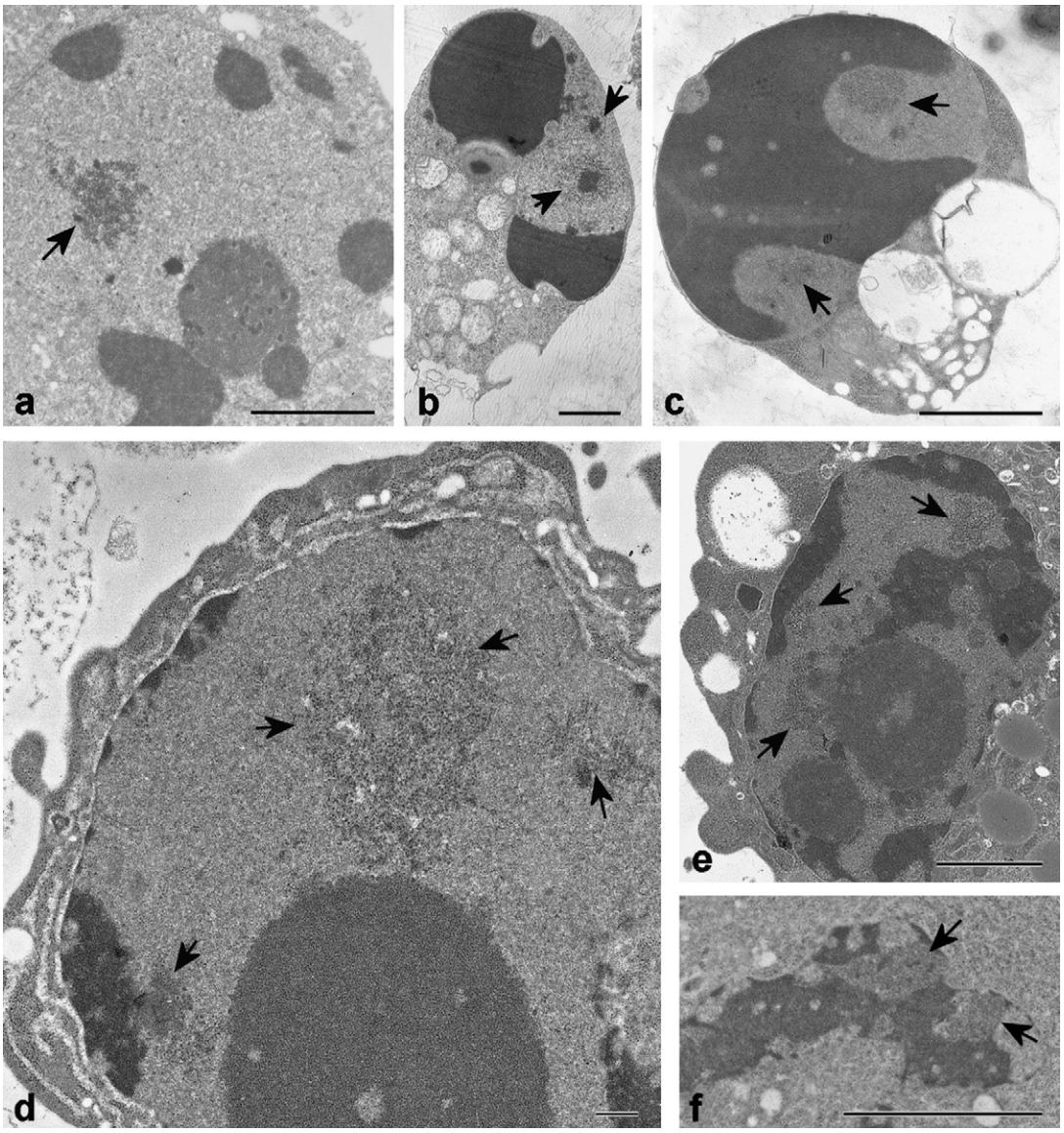

Fig. 1 – Electron micrographs of apoptotic cells after glutaraldehyde-osmium fixation, uranyl acetate–lead citrate staining. All the bars are for 1 μm. (a) Early apoptotic HeLa cells spontaneously occurring in an aged culture: although chromatin condensation is only incipient, the RNP components are already aggregated to form heterogeneous clusters (arrow). (b) Spontaneous apoptosis in a rat thymocyte with chromatin condensed against the nuclear envelope: in the interchromatin areas, the clusters of RNPs (arrows) became more prominent. (c) Apoptotic rat thymocyte after etoposide treatment: extensive chromatin condensation has occurred and large HERDS are visible in the interchromatin space (arrows). (d) Early apoptotic HeLa cells after photosensitization with rose Bengal acetate and irradiation with green light: here too, large fibrogranular clusters of RNPs (arrows) are observed in the nucleus while chromatin starts condensing along the nuclear envelope. (e and f) Apoptotic HeLa cells after treatment with actinomycin D (1 μg/ml for 15 h): HERDS (arrows) increase in size with progressive chromatin condensation, and are always found in the interchromatin space.

tion), middle (crescent-like chromatin condensation) and late (karyorrhexis) apoptosis, to describe the dynamic reorganization of nuclear RNPs and of other nuclear proteins.

In all the experimental conditions considered in this investigation, chromatin condensation and karyorrhexis were found to take place in apoptotic cells, even when DNA degradation into oligonucleosome-sized fragments did not occur (as in HeLa cells photosensitized with rose Bengal acetate, where high-molecular-weight DNA fragments were only found: [43]).

As a general feature, we observed that already at early apoptotic stages chromatin and the RNP-containing structures

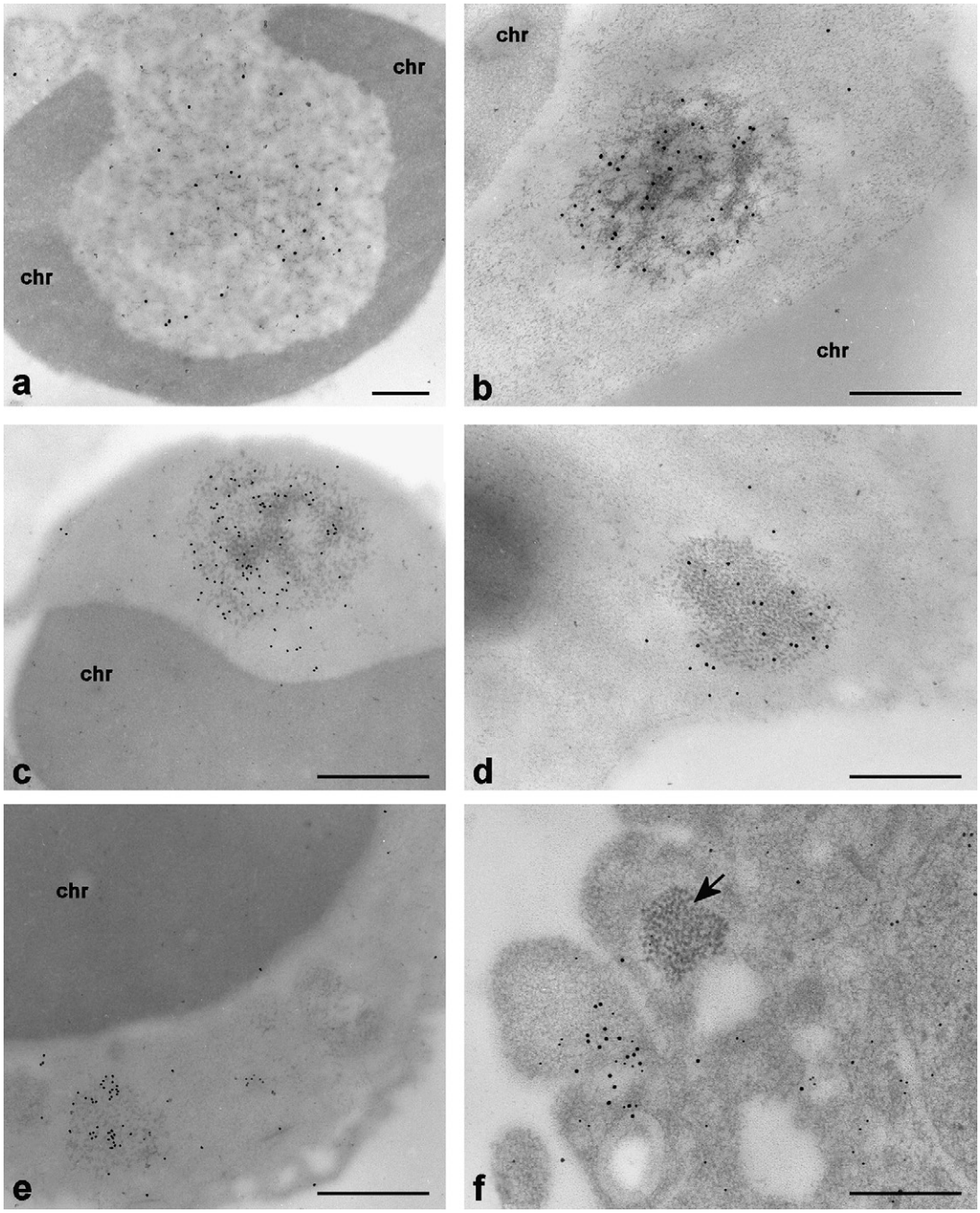

Fig. 2 – Electron micrographs of apoptotic cells after gold immunolabeling and EDTA regressive staining, in formaldehyde–glutaraldehyde fixed samples. All the bars are for 0.5 μm. (a and b) Etoposide-induced apoptotic thymocytes with crescent-shaped condensed chromatin (chr); the gold-immunolabeling for the Sm antigen marks ectopic PF which aggregated in the interchromatin space (a); a dense intranuclear fibrogranular cluster of RNPs is also labelled (b). (c) Spontaneously apoptotic thymocyte: the gold-immunolabeling for hnRNPs marks an ectopic RNP aggregate in the cytoplasm. (d and e) Etoposide-induced apoptotic thymocytes: cytoplasmic HERDS containing large clusters of IG are labelled for the Sm antigen (d) and for hnRNPs (e). (f) Late apoptotic HeLa cells after treatment with actinomycin D (1 μg/ml for 15 h): the cleavage factors CStF (6 nm gold grains) and CFI_m (12 nm gold grains), and a large cluster of IG are released inside separate blebs (arrow).

separate, the latter forming fibro-granular clusters (Fig. 1, panels a–c) which become larger and more heterogeneous, with increasing chromatin condensation (Fig. 1, panels d–f): this was found to occur both in spontaneous and induced apoptotic cells, consistent with previous observations in vivo and in vitro [26,31,32,37].

Irrespective of the cell system, the immunolabeling experiments showed that the heterogeneity depends on the co-localization of specific RNP components which aggregate to form heterogeneous ectopic RNP-derived structures we called HERDS [44] (Fig. 2, panels a–e). This definition refers, in general, to aggregates in which at least two antigens, usually not associated, coexist.

Both in spontaneous apoptotic cells and after all the apoptogenic stimuli used, PF (which are immunopositive for the Sm antigen) move to the interchromatin space (Fig. 2a), become clustered (Fig. 2b), and aggregate to a core of IG to whom both nucleolar and/or non-nucleolar components are eventually added.

Starting from middle apoptosis, HERDS move from the nucleus to the cytoplasm (Fig. 2, panels c–e); here, they maintain their ultrastructural appearance and can still be immunolabeled even in the apoptotic bodies, which are released at the cell surface, in the late apoptotic phase. This demonstrates that the protein components of these RNP-containing aggregates may have been only partially (or even not) cleaved. It is worth noting that several RNPs and non-RNP factors involved in the early events of pre-mRNA processing (such as Sm and SC-35) have been found in nuclear and cytoplasmic HERDS, whereas RNA polymerase II (not shown) or the cleavage factors CFI_m and CStF (Fig. 2f) do not co-locate therein and may be released inside separate blebs.

Many chromatin- or RNP-associated proteins have already been reported to move from the nucleus to the cytoplasm, during apoptosis [11,45]; here we investigated, by a series of dual-immunolabeling experiments, whether RNPs or RNP-associated proteins leave the nucleus at different times. To estimate, on a relative basis, the timing of the nuclear exit of these proteins, we also associated the immunolabeling for one of the nuclear proteins studied with the immunodetection of p89, i.e. the 89 kDa proteolytic fragment of PARP-1 which originates from the cleavage by caspase 3 (for a review see Ref. [46]): as we already described [29], during early apoptosis p89 is generated in the interchromatin space and, in middle apoptosis, it moves to the cytoplasm to be finally found inside the blebs, in late apoptotic phase.

Fig. 3a shows that the non-RNP splicing factor SC35 moves to the cytoplasm when p89 is still located in the nucleus; only in late apoptosis both SC-35 and p89 are found in the cytoplasm as separate clusters (Fig. 3a′) and may eventually reach the apoptotic blebs. Also the hnRNP-associated protein, U2AF65 moves from the nucleus to the cytoplasm before p89 (Fig. 3b), although both proteins are found in apoptotic blebs in karyorrhexic cells (Fig. 3b′).

Thus, nuclear proteins that are found in the interchromatin space during early apoptosis may leave the nucleus with different kinetics. This is the case for SC-35 and fibrillarin (Fig. 3, panels c–c‴) and even for proteins which co-locate in the nucleolus of non-apoptotic cells, i.e. fibrillarin and phosphorylated c-Myc, which exit the nucleus of apoptotic cells and move to the cell surface at different times (Fig. 3, panels d and d′; see also Ref. [41]).

4. Discussion

It is a widely accepted view that chromatin condensation is a primary nuclear sign and a univocal hallmark of apoptosis, as a direct consequence of the proteolytic cleavage of structural proteins (such as histones, matrix components, and lamins) and the activation of endogenous nucleases [10,47]. The results of the present investigation demonstrate that the reorganization of nuclear RNP-containing structures is an apoptotic event occurring much earlier.

Actually, HERDS were observed to form in nuclei with only incipient chromatin condensation as the result of the ectopic coalescence of proteins which usually have specific location in the interchromatin domain (in PF, IG, PG, or the nucleolus). The evidence at electron microscopy suggests that HERDS start forming around a cluster of IG, through the addition of a variety of proteins which are involved in transcription and RNA processing in non-apoptotic cells, such as the Sm-antigen-bearing snRNPs or the non-RNP splicing factor SC35; these protein factors are not cleaved by apoptosis-activated proteases, as much as other nuclear and nucleolar proteins [41,48,49], whereas many other nuclear RNPs undergo degradation (such as U1-70-kDa snRNP and the 60S acidic ribosomal protein P_0 [50]; the ribosomal proteins S15, P0, L5, L6, L36a, L41 [51]; and hnRNPs [52]).

HERDS were also found to form in the nucleus whenever RNA synthesis is either physiologically or experimentally arrested, such as under hypometabolic conditions (hibernation of dormice or lizards [53,54]), or during spermiogenesis and erythrocyte maturation in mammals (reviewed in Ref. [52]), or after treatment with transcription-blocking drugs, such as actinomycin D or DRB ([30] and personal observation). Nuclear HERDS have, therefore, been proposed as a hallmark of transcriptional arrest [52,55] and would represent temporal or terminal storage sites for protein factors involved in the processing of RNAs. In apoptotic cells, HERDS indicate that the RNA processing machinery is dismantled already in early apoptosis, and this may have the adaptive role of blocking the downstream protein synthesis in cells which are committed to die.

Although the molecular mechanisms responsible for RNP restructuring are far from being elucidated, it is tempting to speculate that degradation of RNAs might be involved. Actually, it has been reported that the total amount of RNA decreases in apoptotic cells [56,57] and different apoptosis-activated RNA-degrading nucleases have been described, among which RNase L [58] and endonuclease G, a non-specific DNA/RNA nuclease which migrates from the mitochondrial intermembrane space to the nucleus where it preferentially cleaves RNA and ssDNA, over dsDNA [59]. All these RNA-degrading enzymes could effectively cleave RNA at the transcription sites, i.e. at the level of PF. Under normal conditions, a balance exists between the amount of nascent RNAs and the quantity of the associated proteins which are needed for their processing: we hypothesize that, whenever

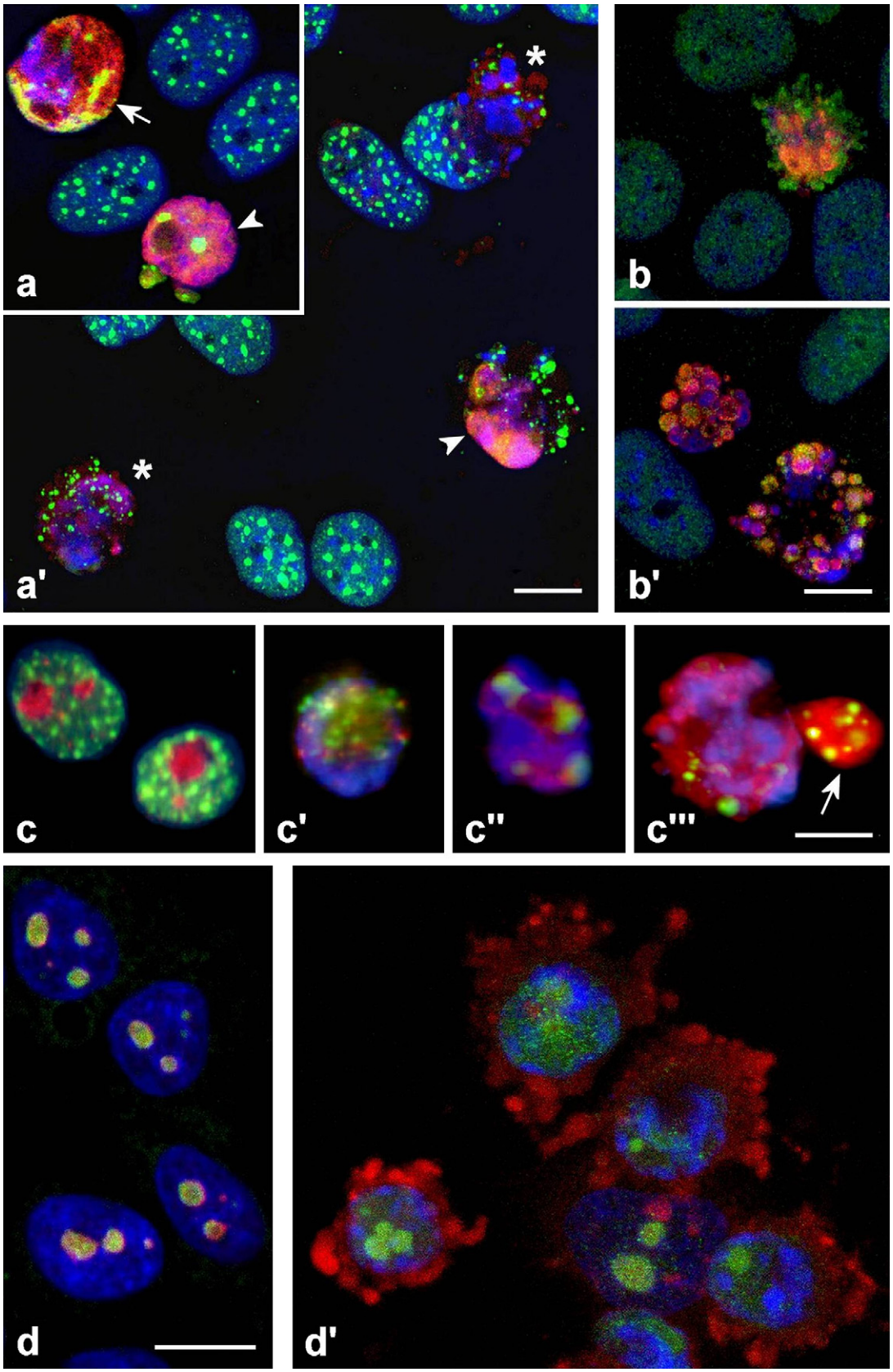

Fig. 3 – Multicolor-fluorescence micrographs of immunolabeled HeLa cells, after apoptogenic treatments. DNA was counterstained with Hoechst 33258. All the bars are for 10 μm. (a and a′) Dual-immunolabeling for p89 (red fluorescence) and the non-RNP splicing factor SC-35 (green fluorescence) in HeLa cells after treatment with actinomycin D (0.1 μg/ml for 15 h). In early apoptotic cells (arrow, in a), p89 and SC-35 colocate in the nucleus, in a diffuse form, whereas in middle

the amount of newly formed hnRNA significantly decreases, RNP proteins (which have a relatively long half-life [60,61]) become exceedingly predominant and accumulate in HERDS, as in a final storage-site. The apoptotic cleavage of newly synthesized RNA would thus provoke a cascade effect leading to the ectopic clustering of different nuclear RNP-containing structures which are directly involved in the transcription and early processing of nuclear RNAs. However, several nuclear proteins which undergo relocation in apoptotic cells do not aggregate in HERDS: for instance, the p89 fragment of PARP-1, phosphorylated c-Myc [41], CENP-B [42], or the cleavage factors CFI and CStF which play a role in late pre-mRNA processing [40].

The majority of nuclear proteins that are not cleaved by apoptotic proteases do leave the nucleus and are detected in the cytoplasm of apoptotic cells. Recently, Schiller et al. [62] showed that translocation to the cytoplasm (and even to the apoptotic blebs) of the ribonuclear protein La/SSB largely precedes DNA degradation and chromatin condensation: this confirms that the reorganization of the interchromatin domain which involves HERDS formation, is an early apoptotic event taking place before the activation of caspases. This is consistent with the evidence that HERDS are also formed whenever transcription is depressed, such as in hibernation or after treatment with RNA-polymerase-blocking agents [52–55]. On the contrary, the massive extrusion of HERDS and of several other nuclear proteins into the cytoplasm during middle and late apoptosis requires caspase activity, since it depends on the irreversible degradation of the architecture and the functional properties of the nuclear envelope.

The export of HERDS to the cytoplasm is, at least in part, an active mechanism. This was demonstrated by experiments on mouse thymocytes induced to apoptosis by etoposide and then treated with 2-deoxyglucose and sodium azide for 30 min, to block ATP synthesis [27]. We found that in ATP-depleted cultures the number of thymocytes showing HERDS in the cytoplasm and blebs decreased, in comparison with thymocytes treated with etoposide only. Interestingly enough, the segregation of the nucleolar components and their association to HERDS are also decreased, suggesting that these processes too need energy to take place.

The transit from the nucleus to the cytoplasm does not occur simultaneously for all the protein or nucleoprotein complexes during apoptosis, and this probably reflects the timing of their dissociation from chromatin or the interchromatin domain, in the course of nuclear condensation.

Our results also confirm that apoptotic blebs may be largely heterogeneous and contain different molecular species of nuclear origin: this is consistent with the reports by other authors [63–66], although a convincing mechanistic explanation is still lacking for the different routes which are apparently travelled by all these molecules (proteins, nucleic acids, nucleoprotein aggregates).

Phagocytosis of apoptotic bodies is the final step of apoptosis, which normally results in the safe disposal of cell remnants [4,6]: provided that the removal of apoptotic cells is rapid and effective, the half-life of dying cells in the tissues is relatively short. This makes it likely that developing lymphocytes (which reside in organs where the clearance mechanisms are highly effective) will not encounter late apoptotic products. In this way, tolerance towards late apoptotically modified molecules cannot be usually established. In the presence of defective phagocytosis, or when massive apoptosis overwhelms the clearance capability of the tissue scavenger cells, the disposal of apoptotic remnants becomes insufficient and unphagocytosed late apoptotic cells may accumulate in the tissue where they may be engulfed by antigen-presenting (such as dendritic) cells [67,68]; an autoimmune response may thus be elicited, by which apoptosis-derived auto-antigens are recognized and presented to the immune system. Actually, apoptotic cells have been suggested as a powerful source of potential auto-antigens, which are produced through the partial cleavage, by caspases or nucleases, of numerous cytoplasmic and nuclear substrates [48,64,65]. In fact, autoimmunity requires an appropriate genetic background to take place and can be explained not only by the failure to eliminate auto-reactive cells, but also by a correct immune response against ''new'' antigens deriving from apoptotically modified cellular components (such as those aggregating in HERDS) which thus acquire non-self characteristics [69].

4.1. *Concluding remarks: a few answers and some open questions*

The ectopic reorganization of nuclear RNP-containing structures in HERDS takes place during spontaneous and induced apoptosis; this occurs before chromatin condensation, and should therefore be seen as an early apoptotic nuclear

apoptosis SC-35 already moves to the cytoplasm (arrowheads, in a and a′); the release of p89 to the cytoplasm takes place very late, in karyorrhexic cells (asterisks, in a′). (b and b′) HeLa cells after treatment with actinomycin D (0.1 μg/ml for 15 h); (b): hnRNPs (green fluorescence) are released from the nucleus before p89 (red fluorescence); (b′) in late apoptosis, p89 and hnRNPs may colocate in apoptotic blebs. (c–c‴) Dual-immunolabeling for the nucleolar protein, fibrillarin (red fluorescence) and the non-RNP splicing factor SC-35 (green fluorescence) in HeLa cells treated with etoposide (50 μM for 15 h). In non-apoptotic cells (c), fibrillarin locates in the nucleoli whereas SC-35 exhibits the typical spotted pattern; in early apoptotic cells, fibrillarin redistributes through the nucleus in a diffuse form (c′) and then moves to the cytoplasm since middle apoptosis (c″ and c‴); this relocation also occurs for SC-35, which, however, forms clusters, first in the nucleus then in the cytoplasm. Both proteins are released in apoptotic blebs (arrow in c‴). (d and d′) Dual-immunolabeling for P-c-Myc (green fluorescence) and fibrillarin (red fluorescence) in HeLa cells treated with actinomycin D (0.1 μg/ml for 15 h). In non-apoptotic cells (d) these proteins colocate in the nucleoli; during apoptosis, both P-c-Myc and fibrillarin diffuse from the nucleolus, but they are released from the nucleus with a different timing, fibrillarin passing into the cytoplasm when P-c-Myc is still nuclear (d′).

hallmark, which can be detected morphologically by electron microscopy, and immunocytochemically by fluorescence microscopy. HERDS relocate to the cytoplasm from middle apoptosis, move to the cell surface, and are found inside the blebs and apoptotic bodies, where they can still be immunolabeled. Their heterogeneous composition and apparently partial proteolytic degradation may account for their role in the etiology of autoimmune diseases. Similarly, other nuclear proteins (such as phosphorylated c-Myc and the p89 fragment of PARP-1) move, in a partially degraded form, to the cytoplasm but do not associate with HERDS; their exit from the nucleus takes place at different times during apoptosis and they often do not co-locate when released inside membrane-limited blebs.

Some major points need, however, further investigation. There is suggestive indication that an early degradation of nascent RNAs could be the ''primum movens'' in the process of HERDS formation, having at the same time a causal effect in the blockade of protein synthesis during apoptosis: this should have an obviously adaptive role in the process of regulated cell death, but still requires a direct experimental evidence.

As already recalled, HERDS were also found under different conditions of transcriptional arrest (such as hibernation or spermiogenesis) where they become storage sites for transcription and splicing factors: as soon as transcription is resumed (when the hibernator awakes or fertilization occurs), these protein factors relocate to their usual nuclear RNP-containing structures (PF and IG) and, consequently, HERDS disappear. It cannot be excluded that also during apoptosis HERDS formation represents the usual defence mechanism operated by the cell, which becomes irreversible when the nuclear structure is irreparably damaged, i.e. when the nuclear lamina and envelope structures are degraded, and the interchromatin contents can leak into the cytoplasm. This also needs to be experimentally proved.

Finally, it has recently been suggested that the reorganization of the vacuolar system (namely the endoplasmic reticulum and the Golgi apparatus) and, especially, the actomyosin and microtubule cytoskeleton may play a major role in the intracellular displacement of organelles and in bleb formation: in particular, small blebs form relatively early in apoptosis (before DNA degradation had occurred) whereas larger blebs (which contain remnants of the endoplasmic reticulum and chromatin) are extruded in later stages only [70,71]. The rearrangement and/or the active movement of the actomyosin and microtubule cytoskeleton, and the different timing in the release from the nucleus of molecules or molecular aggregates can account for the large heterogeneity in size and content of apoptotic blebs which has been repeatedly reported in the literature [50,52,63,66,70], but again a mechanistic explanation for these phenomena is still lacking.

Acknowledgements

We whish to thank Prof. Christine Milcarek for providing us with the antibody recognizing CStF, and Dr. Claudia Alpini for the kind gift of autoimmune sera. The technical assistance of Mrs. Paola Veneroni is also acknowledged. This research was partially supported by the University of Pavia (FAR 2007), Fondazione Cariplo (2006-0734) and Regione Lombardia (Project Metadistretti n. 4238).

REFERENCES

[1] Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239–57.
[2] Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell 2004;116:205–19.
[3] Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251–306.
[4] Savill J, Fadok V. Corpse clearance defines the meaning of cell death. Nature 2000;407:784–8.
[5] Dini L. Phagocyte and apoptotic cell interplay. In: Scovassi AI, editor. Apoptosis. Kerala, India: Research Signpost; 2005. p. 131–44.
[6] Ravichandran KS, Lorenz U. Engulfment of apoptotic cells: signals for a good meal. Nat Rev Immunol 2007;7:964–74.
[7] Kumar S. Caspase function in programmed cell death. Cell Death Differ 2007;14:32–43.
[8] Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol 2008;9:231–41.
[9] Scovassi AI, Torriglia A. Activation of DNA-degrading enzymes during apoptosis. Eur J Histochem 2003;47:185–94.
[10] Samejima K, Earnshaw WC. Trashing the genome: the role of nucleases during apoptosis. Nat Rev Mol Cell Biol 2005;6:677–88.
[11] Ferrando-May E. Nucleocytoplasmic transport in apoptosis. Cell Death Differ 2005;12:1263–76.
[12] Tembe V, Henderson BR. Protein trafficking in response to DNA damage. Cell Signal 2007;19:1113–20.
[13] van Driel R, Fransz PF, Verschure PJ. The eukaryotic genome: a system regulated at different hierarchical levels. J Cell Sci 2003;116:4067–75.
[14] Fakan S. Ultrastructural cytochemical analyses of nuclear functional architecture. Eur J Histochem 2004;48:5–14.
[15] Cremer T, Cremer C. Rise, fall and resurrection of chromosome territories: a historical perspective. Part I. The rise of chromosome territories. Eur J Histochem 2006;50:161–76.
[16] Cremer T, Cremer C. Rise, fall and resurrection of chromosome territories: a historical perspective. Part II. Fall and resurrection of chromosome territories during the 1950s to 1980s. Part III. Chromosome territories and the functional nuclear architecture: experiments and models from the 1990s to the present. Eur J Histochem 2006;50:223–72.
[17] Bernhard W. A new staining procedure for electron microscopical cytology. J Ultrastruct Res 1969;27:250–65.
[18] Monneron A, Bernhard W. Fine structural organization of the interphase nucleus in some mammalian cells. J Ultrastruct Res 1969;27:266–88.
[19] Fakan S, Bernhard W. Localisation of rapidly and slowly labelled nuclear RNA as visualized by high resolution autoradiography. Exp Cell Res 1971;67:129–41.
[20] Fakan S, Bernhard W. Nuclear labelling after prolonged 3H-uridine incorporation as visualized by high resolution autoradiography. Exp Cell Res 1973;79:431–44.
[21] Spector DL, Fu XD, Maniatis T. Associations between distinct pre-messenger RNA splicing components and the cell nucleus. EMBO J 1991;10:3467–81.
[22] Fakan S. Perichromatin fibrils are in situ forms of nascent transcripts. Trends Cell Biol 1994;4:86–90.
[23] Cmarko D, Verschure PJ, Martin TE, Dahmus ME, Krause S, Fu XD, et al. Ultrastructural analysis of transcription and

splicing in the cell nucleus after bromo-UTP microinjection. Mol Biol Cell 1999;10:211–23.
[24] Misteli T, Spector DL. The cellular organization of gene expression. Curr Opin Cell Biol 1998;10:323–31.
[25] Puvion E, Puvion-Dutilleul F. Ultrastructure of the nucleus in relation to transcription and splicing: roles of perichromatin fibrils and interchromatin granules. Exp Cell Res 1996;229:217–25.
[26] Biggiogera M, Bottone MG, Pellicciari C. Nuclear ribonucleoprotein-containing structures undergo severe rearrangement during spontaneous thymocyte apoptosis. A morphological study by electron microscopy. Histochem Cell Biol 1997;107:331–6.
[27] Biggiogera M, Cisterna B, Bottone, Soldani C, Pellicciari C. Nuclear RNP and nucleolar-associated proteins during apoptosis: a politically correct form of segregation? Dynam Cell Biol 2007;1:65–71.
[28] Pellicciari C, Manfredi AA, Bottone MG, Schaack V, Barni S. A single-step staining procedure for the detection and sorting of unfixed apoptotic thymocytes. Eur J Histochem 1993;37:381–90.
[29] Soldani C, Lazzè MC, Bottone MG, Tognon G, Biggiogera M, Pellicciari CE, et al. Poly(ADP-ribose) polymerase cleavage during apoptosis: when and where? Exp Cell Res 2001;269:193–201.
[30] Fraschini A, Bottone MG, Scovassi AI, Denegri M, Risueño MC, Testillano PS, et al. Changes in extranucleolar transcription during actinomycin D-induced apoptosis. Histol Histopathol 2005;20:107–17.
[31] Pellicciari C, Bottone MG, Scovassi AI, Martin TE, Biggiogera M. Rearrangement of nuclear ribonucleoproteins and extrusion of nucleolus-like bodies during apoptosis induced by hypertonic stress. Eur J Histochem 2000;44:247–54.
[32] Biggiogera M, Scherini E, Mares V. Ultrastructural cytochemistry of *cis*-dichlorodiammine platinum-II induced apoptosis in immature rat cerebellum. Acta Histochem Cytochem 1990;23:831–9.
[33] Bottone MG, Soldani C, Veneroni P, Avella D, Pisu M, Bernocchi G. Cell proliferation, apoptosis and mitochondrial damage in rat B50 neuronal cells after cisplatin treatment. Cell Prolif 2008;41:506–20.
[34] Soldani C, Croce AC, Bottone MG, Fraschini A, Biggiogera M, Bottiroli G, et al. Apoptosis in tumor cells photosensitized with rose Bengal acetate is induced by multiple organelle photodamage. Histochem Cell Biol 2007;128:485–95.
[35] Bottone MG, Soldani C, Tognon G, Gorrini C, Lazzè MC, Brison O, et al. Multiple effects of paclitaxel are modulated by a high c-myc amplification level. Exp Cell Res 2003;290:49–59.
[36] Bottone MG, Soldani C, Fraschini A, Alpini C, Croce AC, Bottiroli G, et al. Enzyme-assisted photosensitization with rose Bengal acetate induces structural and functional alteration of mitochondria in HeLa cells. Histochem Cell Biol 2007;127:263–71.
[37] Biggiogera M, Bottone MG, Martin TE, Uchiumi T, Pellicciari C. Still immunodetectable nuclear RNPs are extruded from the cytoplasm of spontaneously apoptotic thymocytes. Exp Cell Res 1997;4:512–20.
[38] Ismaïli N, Pérez-Morga D, Walsh P, Mayeda A, Pays A, Tebabi P, et al. Characterization of a SR protein from Trypanosoma brucei with homology to RNA-binding *cis*-splicing proteins. Mol Biochem Parasitol 1999;102:103–15.
[39] Oliveri I, Cisterna B, Flach F, Barabino SML, Cecconi F, Battistelli S, et al. Temporary depression of transcription in mouse pre-implantion embryos from mice fed on genetically modified soybean. In: Proceedings of the forty-eighth symposium of the society for histochemistry; 2006. p. 35.
[40] Cardinale C, Cisterna B, Bonetti P, Aringhieri C, Biggiogera M, Barabino SML. Subnuclear localization and dynamics of the pre-mRNA 3′ end processing factor CF Im68. Mol Biol Cell 2007;18:1282–92.
[41] Soldani C, Bottone MG, Pellicciari CE, Scovassi AI. Nucleolus disassembly in mitosis and apoptosis: dynamic redistribution of phosphorylated c-Myc, fibrillarin and Ki-67. Eur J Histochem 2006;50:273–80.
[42] Perdoni F, Bottone MG, Soldani C, Veneroni P, Alpini C, Pellicciari C, et al. Distribution of centromeric proteins and PARP-1 during mitosis and apoptosis. In: Proceedings of the apoptosis world 2008: from mechanisms to applications; 2008. p. 403.
[43] Soldani C, Bottone MG, Croce AC, Fraschini A, Perdoni F, Bottiroli G, et al. Photosensitization with rose Bengal acetate induces apoptosis in tumor cells without oligonucleosome DNA degradation. In: Proceedings of the eighth multinational congress of microscopy; 2007. p. 513–4.
[44] Biggiogera M, Bottone MG, Pellicciari C. Nuclear RNA is extruded from apoptotic cells. J Histochem Cytochem 1998;46:999–1005.
[45] Pellicciari C, Bottone MG, Soldani C, Vecchio L, Biggiogera M. Extrusion of nuclear proteins during apoptosis. In: Scovassi AI, editor. Apoptosis. Kerala, India: Research Signpost; 2005. p. 131–44.
[46] Soldani C, Scovassi AI. Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. Apoptosis 2002;7:321–8.
[47] Parrish JZ, Xue D. Cuts can kill: the roles of apoptotic nucleases in cell death and animal development. Chromosoma 2006;15:89–97.
[48] Rosen A, Casciola-Rosen L. Autoantigens as substrates for apoptotic proteases: implications for the pathogenesis of systemic autoimmune disease. Cell Death Differ 1999;6:6–12.
[49] Fischer U, Janicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ 2003;10:76–100.
[50] Casciola-Rosen LA, Miller DK, Anhalt GJ, Rosen A. Specific cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein is a characteristic biochemical feature of apoptotic cell death. J Biol Chem 1994;269:30757–60.
[51] Nishida J, Shiratsuchi A, Nadano D, Sato TA, Nakanishi Y. Structural change of ribosomes during apoptosis: degradation and externalization of ribosomal proteins in doxorubicin-treated Jurkat cells. J Biochem 2002;131:485–93.
[52] Biggiogera M, Bottone MG, Scovassi AI, Soldani C, Vecchio L, Pellicciari C. Rearrangement of nuclear ribonucleoprotein (RNP)-containing structures during apoptosis and transcriptional arrest. Biol Cell 2004;96:603–15.
[53] Malatesta M, Zancanaro C, Martin TE, Chan EKL, Amalric F, Luhrmann R, et al. Cytochemical and immunocytochemical characterization of nuclear bodies during hibernation. Eur J Cell Biol 1994;65:82–93.
[54] Malatesta M, Frigato E, Baldelli B, Battistelli S, Foà A, Bertolucci C. Influence of temperature on the liver circadian clock in the ruin lizard *Podarcis sicula*. Microsc Res Tech 2007;70:578–84.
[55] Biggiogera M, Pellicciari C. Heterogeneous ectopic RNP-derived structures (HERDS) are markers of transcriptional arrest. FASEB J 2000;14:828–34.
[56] Cidlowsky JA. Glucocorticoids stimulate ribonucleic acid degradation in isolated rat thymic lymphocytes in vitro. Endocrinology 1982;111:184–90.
[57] Delic J, Coppey-Moisant M, Magdalenat H. Gamma-ray induced transcription and apoptosis-associated loss of 28S rRNA in interphase human lymphocytes. Int J Radiat Biol 1993;64:39–46.

[58] Bisbal C, Silverman RH. Diverse functions of RNase L and implications in pathology. Biochimie 2007;89:789–98.
[59] Widlak P, Li LY, Wang X, Garrard WT. Action of recombinant human apoptotic endonuclease G on naked DNA and chromatin substrates: cooperation with exonuclease and DNase I. J Biol Chem 2001;276:48404–9.
[60] Martin TE, Jones R, Billings P. hnRNP core proteins: synthesis, turnover and intracellular distribution. Mol Biol Rep 1979;5:37–42.
[61] Martin TE, Okamura CS. Immunocytochemistry of nuclear hnRNP complexes. In: Busch H, editor. The cell nucleus, vol.9. New York: Academic Press; 1981. p. 119–44.
[62] Schiller M, Bekeredjian-Ding I, Heyder P, Blank N, Ho AD, Lorenz HM. Autoantigens are translocated into small apoptotic bodies during early stages of apoptosis. Cell Death Differ 2008;15:183–91.
[63] Halicka HD, Bedner E, Darzynkiewicz Z. Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. Exp Cell Res 2000;260:248–56.
[64] Cline AM, Radic MZ. Murine lupus autoantibodies identify distinct subsets of apoptotic bodies. Autoimmunity 2004;37:85–93.
[65] Cline AM, Radic MZ. Apoptosis, subcellular particles, and autoimmunity. Clin Immunol 2004;112:175–82.
[66] Dieker J, Cisterna B, Monneaux F, Decossas M, van der Vlag J, Biggiogera M, et al. Apoptosis-linked changes in the phosphorylation status and subcellular localization of the spliceosomal autoantigen U1-70K. Cell Death Differ 2008;15:793–804.
[67] Manfredi AA, Iannacone M, D'Auria F, Rovere-Querini P. The disposal of dying cells in living tissues. Apoptosis 2002;7:153–61.
[68] Frisoni L, McPhie L, Colonna L, Sriram U, Monestier M, Gallucci S, et al. Nuclear autoantigen translocation and autoantibody opsonization lead to increased dendritic cell phagocytosis and presentation of nuclear antigens: a novel pathogenic pathway for autoimmunity? J Immunol 2005;175:2692–701.
[69] Kaplan M. Apoptosis in systemic lupus erythematosus. Clin Immunol 2004;112:210–8.
[70] Lane JD, Allan VJ, Woodman PG. Active relocation of chromatin and endoplasmic reticulum into blebs in late apoptotic cells. J Cell Sci 2005;118:4059–71.
[71] Moss DK, Lane JD. Microtubules: forgotten players in the apoptotic execution phase. Trends Cell Biol 2006;16:330–8.