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Nuclear RNP and Nucleolar-Associated Proteins during Apoptosis: a Politically Correct Form of Segregation?

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ABSTRACT

During apoptosis, a major rearrangement of nuclear RNP-containing structures takes place, in parallel with chromatin changes. In the interchromatin space, nucleoplasmic RNP constituents (perichromatin fibrils and granules, and interchromatin granules) as well as the nucleolar components aggregate into heterogeneous clusters we called HERDS (for Heterogeneous Ectopic RNP-Derived Structures). In late apoptosis, these aggregates are extruded from the nucleus and released at the cell surface within apoptotic bodies; we also confirm that during apoptosis nucleolar proteins (i.e phosphorylated c-Myc, Ki-67, fibrillarin) already segregate in the nuclei and remain separated in the cytoplasm. Immunocytochemical evidence from light and electron microscopy also demonstrate that the sub-cellular particles in the apoptotic bodies may be heterogeneous in size and content, and may still include immunodetectable nuclear proteins and nucleic acids. Remarkably, the extrusion from the nucleus of a wide and heterogeneous spectrum of proteins which survive in a partially degraded (or even in an undegraded) form during the late steps of apoptosis legitimizes the growing interest toward those *novel* and ectopic molecular complexes which may play a role in the etiology of autoimmune diseases. In this paper we also confirm that the formation of HERDS is a more general phenomenon which may be triggered by transcriptional arrest.

Keywords: electron microscopy, HERDS, immunocytochemistry, nuclear ribonucleoproteins, transcription

CONTENTS

RNP COMPONENTS OF THE CELL NUCLEUS

The replication and transcription events which take place inside the cell nucleus have their morphological counterpart, as already demonstrated by Bernhard and co-workers using electron microscopy analysis (Bernhard 1969; Monneron and Bernhard 1969; Fakan and Bernhard 1971, 1973). By means of an ultrastructural technique based on treatment with EDTA, condensed chromatin may be bleached whereas the RNP-containing structures in the interchromatin space become more contrasted. Besides the nucleolus, the most important among these components are perichromatin fibrils, perichromatin granules, interchromatin granules, Cajal bodies and several other structures.

Perichromatin fibrils (PF) represent the structural sites where transcription takes place: they can be easily recognized in electron microscopy as irregularly shaped, heterogeneous structures being present at the periphery of condensed chromatin (**Fig. 1A**). PF contain RNA, associated with hnRNPs and snRNPs (Fakan *et al*. 1984) and are the sites of both hnRNA transcription and co-transcriptional splicing (Fakan *et al*. 1984; Cmarko *et al*. 1999). In addition to hnRNPs and snRNPs (Fakan *et al*. 1984), the non-snRNP SC35 splicing factor (Spector *et al*. 1991) and several serine-arginine–rich proteins and cleavage factors were also found in PF (Cardinale *et al*. 2007). In recent years, the role of PF in transcription has been questioned based on immunocytochemical investigations performed by fluorescence microscopy: using this approach only, the immunopositivity for PF was interpreted as a background signal thus leading to the wrong conclusion that transcription could take place elsewhere (Carter *et al*. 1991). On the contrary, PF must correctly be considered as a very finely tuned marker of transcriptional alterations (Biggiogera *et al*. submitted).

Interchromatin granules (IG) represent a storage site for snRNP and non-snRNP splicing factors (Fakan 1994) and are a possible site for spliceosome assembly (Misteli and Spector 1998). The clusters of IG are also easily recogni-

Received: 28 February, 2007. Accepted: 11 April, 2007.

Invited Mini-Review

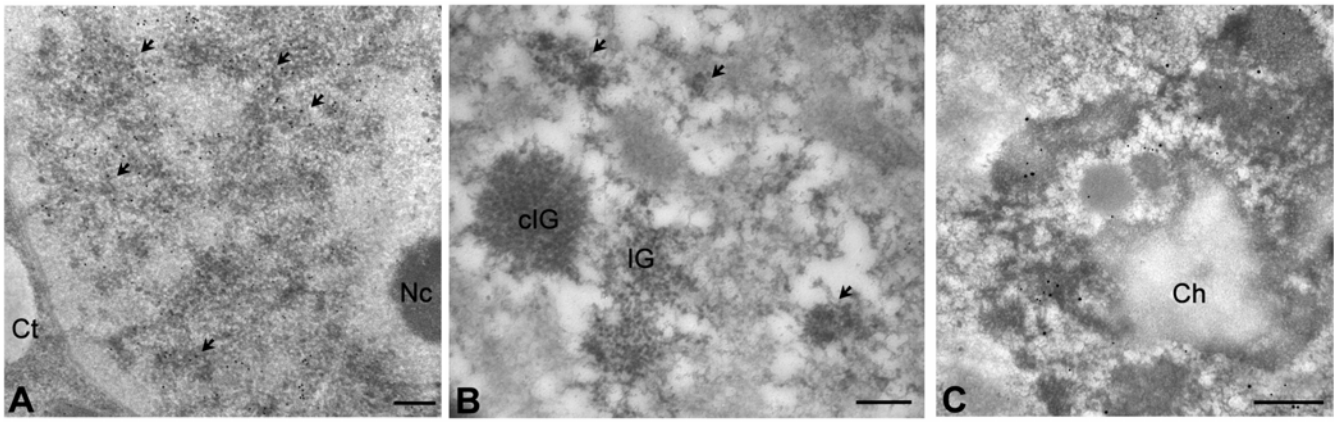

Fig. 1 RNP-containing structures and their segregation in apoptosis. (**A**) HeLa cell, immunolabelling for hnRNP core proteins and EDTA staining for RNP components. The condensed chromatin at the nuclear periphery is bleached, and evident are the heavily labelled perichromatin fibrils (arrowheads) and the unlabelled nucleolus (Nc). Ct, cytoplasm. (**B**) After induction of apoptosis, RNP structural components begin to aggregate: PF (arrowheads), interchromatin granules (IG) and their condensed clusters (cIG) move to form HERDS. (**C**) HERDS formation is complete, with intermingling of nuclear and nucleolar components. After double immunolabelling, HERDS are positive for fibrillarin (small gold grains) and hnRNPs (large grains). Ch, condensed chromatin. Bar = 200 nm.

zable and are characteristically labeled by anti-Sm and anti-PANA antibodies (Clevenger and Epstein 1984; Fakan *et al*. 1984; Leser *et al*. 1989; Spector *et al*. 1991).

Perichromatin granules (PG) are involved in the storage and the nucleus-to-cytoplasm transport of mRNA (Fakan 1994, 2004). Their number is influenced by the metabolic status, like for instance in hibernation (Zancanaro *et al*. 1993) or by drugs (Lafarga *et al*. 1993). It is still not clear whether they contain mRNA or if, in some cases, pre-mRNA can be stored also in this form. Nonetheless, the storage role of these granules is undisputed, and they can exit the nucleus by uncoiling (like in the Balbiani ring granules; Daneholt 2001).

Finally, for Cajal bodies (or coiled bodies, as they were originally called) a transport role has been clarified as well as that of storage of RNA and protein components (Carmo-Fonseca 2002).

The larger RNP-containing nuclear structure is represented by the nucleolus. Its main function, ribosome biogenesis, is morphologically appreciable in the granular component (GC), where the maturing subunits are located prior to their export to the cytoplasm.

Several other RNP-based structures and/or nuclear domains have been described, such as PML bodies (Eskiw *et al*. 2003), which have been proposed to be involved in apoptosis by regulating the availability of apoptotic signal transducers (Hofmann and Will 2003).

LOCATION AND MOVEMENT OF RNP COMPONENTS

The RNP structural components described above have specific intranuclear location: at the periphery of condensed chromatin (PF and PG) and in the so-called interchromatin space (IG). The correct intranuclear location of these RNP-containing particles is a necessary prerequisite for the maturation of nuclear RNAs (for a review, see Puvion and Puvion-Dutilleul 1996). PF and PG, necessarily, move away from their synthetic sites toward the nuclear pores and finally the cytoplasm, while IG are a more or less stable structure, subject only to enlargement after hypertonic stress or viral infection (Fraschini and Fuhrman Conti 1995; Puvion and Puvion-Dutilleul 1996).

The 100 nm thick zone close to the condensed chromatin is called the perichromatin area, and corresponds to the space where active genes are transcribed and DNA replication takes place (Cmarko *et al*. 1999, 2003; Fakan 2004) (**Fig. 1A**).

The transport of the transcription products to the cytoplasm can be achieved by two mechanisms: diffusion and active transport. The first is, so far, the most accepted (Politz *et al*. 2006) both for PF and for ribosomal subunits. However, it must be taken into account that the interchromatin space may be considered as a tunnel, containing nuclear structural components, flanked by condensed chromatin and ending in the pore zone: likely, these structural constraints affect diffusion and can make it at least an anomalous diffusion (Wachsmuth *et al*. 2000). The recent data concerning the role played by motor proteins (namely, nuclear myosin I and actin) in pre-mRNA and pre-rRNA transcription (Nowak *et al*. 1997; Pestic-Dragovich *et al*. 2000; Philimonenko *et al*. 2004) have stimulated further research on other motor protein-driven mechanisms. Cisterna *et al*. (2006) have demonstrated that the export of at least a subset of the small ribosomal subunit is energy-dependent. Therefore an active transport cannot be excluded also for the export of PF and PG; this would represent a supporting mechanism to increase the export of RNPs to the cytoplasm whenever necessary.

NUCLEAR AND NUCLEOLAR STRUCTURES CONTAINING RNPS ARE REARRANGED AND RELOCATE DURING APOPTOSIS TO FORM HETEROGENEOUS ECTOPIC RNP-DERIVED STRUCTURES (HERDS)

The controlled disruption of the whole nuclear structure which occurs during apoptosis characteristically involves chromatin, which undergoes progressive condensation and often collapses against the nuclear envelope in the form of crescents (Earnshaw and Bernat 1995; Kaufmann and Hengartner 2001). In parallel with apoptotic margination and condensation of chromatin, nucleolar and non-nucleolar RNP-containing structures also exhibit dramatic reorganization: this has already been shown in the classical papers by Kerr *et al*. (1972) and Wyllie *et al*. (1980) where segregation and disappearance of the nucleolus have been described; more recently, this was confirmed by us in spontaneous and induced apoptosis in which nucleolar segregation occurs and the complex organization of the organelle is finally lost, although the dense fibrillar component (DFC) and the GC can be still recognized in the interchromatin space (Biggiogera *et al*. 2004).

At early apoptotic stages, also the nucleoplasmic RNPs in PF, PG, IG segregate in the interchromatin space, where they form fibro-granular heterogeneous clusters (**Fig. 1B**) often in association with the nucleolar components; this has been repeatedly observed during apoptosis *in vivo* (Biggiogera *et al*. 1990, 1997) and in *in vitro* cell systems (Biggiogera *et al*. 1997a, 1997b, 1998; Pellicciari *et al*. 2000). Consistently, using immunolabeling experiments with light and electron microscopy we found that during apoptosis specific

RNP protein components co-localize ectopically (**Fig. 1C**). Similar rearrangements of RNPs have been observed by different authors (Lafarga *et al*. 1997; Biggiogera *et al*. 2004) in several mammalian cell systems (among which rat cerebellar cells, thymocytes and C6 glioma cells; mouse 3T3 fibroblasts; human EUE and HeLa cells) following apoptogenic stimuli (hypertonic stress or heat shock; serum deprivation; exposure to drugs inducing either DNA damage or transcription arrest (reviewed in Biggiogera *et al*. 2004). We have called these RNP aggregates *HERDS*, for Heterogeneous Ectopic RNP-Derived Structures (Biggiogera *et al*. 1998) suggesting that their presence could be a nuclear hallmark of both spontaneous and induced apoptosis (Biggiogera and Pellicciari 2000; Biggiogera *et al*. 2004).

It is worth stressing here that Bernhard (1969) had already observed that IG are extremely resistant structures from which RNA could not be removed unless a previous protease treatment had been performed. Such a characteristic makes IG the most obvious candidate for the role of *aggregation core* in the formation of HERDS during apoptosis and transcriptional arrest.

Several nuclear proteins have been investigated for their possible reorganization and cleavage during apoptosis and there is an ever-growing list of proteins which may be substrates for caspases (for a review, see Fischer *et al*. 2003). It has been demonstrated by Western blot analysis that some nuclear proteins, such as hnRNPs, U1-70-kDa snRNP and the 60S acidic ribosomal protein P_0 are cleaved (Casciola-Rosen *et al*. 1994), whereas others (e.g. the antigen Sm, Ro and La: Rosen and Casciola-Rosen 1999, and pers. obs.) are not degraded. The six ribosomal proteins, S15, P0, L5, L6, L36a, L41 have also been shown to be cleaved during apoptosis (Nishida *et al*. 2002). This is consistent with the evidence that, even in late apoptosis, RNPs in the HERDS can often be labeled by specific antibodies against different proteins (Biggiogera *et al*. 1997b) while the immunoreactivity of other proteins is lost.

SEVERAL NUCLEOLAR NON-RNP COMPONENTS ALSO REARRANGE IN APOPTOTIC CELLS

Evidence has been provided that several non-RNP nucleolar proteins actually rearrange and may have different fates, in apoptotic cells.

It has been reported that the nucleolar antigen UBF is cleaved, whereas other nucleolar proteins (such as fibrillarin, B23, C23 and topoisomerase I) are not (Martelli *et al*. 2000; Horky *et al*. 2001; Martelli *et al*. 2001; Horky *et al*. 2002).

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nucleolar enzyme playing the active role of "nick sensor" during DNA repair and apoptosis when it migrates to the chromatin compartment and synthesizes ADP-ribose from NAD^+ in the presence of DNA strand breaks (Soldani and Scovassi 2002). The precise relationship between PARP-1 activation and degradation during apoptosis has long been a matter of debate; we have finally demonstrated (Soldani *et al*. 2001) that poly (ADP-ribose) synthesis is a very early response during apoptosis and that PARP-1 proteolysis by caspases occurs concomitantly.

One of the most studied proteins in the nucleolus, nucleolin has also been reported to be degraded in parallel with PARP-1 cleavage, in UV-induced apoptosis (Mi *et al*. 2003).

Recently, Soldani *et al*. (2006) showed that in apoptotic HeLa cells fibrillarin and phosphorylated c-Myc are not cleaved, whereas the antigen Ki-67 is degraded.

NUCLEAR PROTEINS WHICH ASSOCIATE WITH RNP-CONTAINING STRUCTURES MIGRATE INTO THE CYTOPLASM WHERE THEY RELOCATE INDEPENDENTLY

It has been demonstrated, by means of immunolabeling techniques, that several proteins that are normally located inside the nucleus move into the cytoplasm in late apoptotic cells (irrespective of the apoptogenic stimuli used): this proved to be the case for PARP-1, for nuclear matrix proteins (Zweyer *et al*. 1997; Rosen and Casciola-Rosen 1999; Martelli *et al*. 2001), for some non-snRNP splicing factors (such as SC-35), and for the protein components of hnRNPs, snRNPs and the nucleolus (Biggiogera *et al*. 1997a, 1997b; Rosen and Casciola-Rosen 1999; Biggiogera *et al*. 2004) which aggregate into largely heterogeneous complexes.

The release from the nucleus to the cytoplasm of nuclear proteins may be likely due to the partial disassembling of the nuclear envelope due to the degradation of lamins, although the loss of the nuclear localization signals as the consequence of proteolytic cleavage cannot be excluded (actually, this was demonstrated for La antigen: Ayukawa *et al*. 2000).

In apoptotic EUE cells after hypertonic stress in culture (Pellicciari *et al*. 2000), and in HeLa cells, after treatment with high concentrations of actinomycin D (pers. obs.), morphologically intact nucleoli have been observed in the cytoplasm and even inside apoptotic blebs. These *cytoplasmic* nucleoli retained their normal morphology and it was sometimes possible to demonstrate the presence of DNA at their periphery.

We have recently found by immunofluorescence that some cycle-related nuclear proteins (namely the proliferating cell nuclear antigen, PCNA, and the phosphorylated product of the proto-oncogene *c-myc*) are also extruded into the cytoplasm and may often be detected in cytoplasmic fragments blebbing at the cell surface in late apoptosis (Pellicciari *et al*. 2005; Soldani *et al*. 2006).

RNA IN HERDS

Cytochemical analyses at fluorescence and electron microscopy (Biggiogera *et al*. 1998) demonstrated that nuclear HERDS always contain appreciable amounts of RNAs of different origins: HERDS were labeled both by anti-hnRNP and anti-snRNP antibodies (Biggiogera *et al*. 1997a), and therefore a part of the cytochemically detectable RNA should derive from the PF (i.e. the sites of extranucleolar transcription: Fakan 1994), the IG (where snRNAs are present) and the PG (constituted by mRNAs and, possibly, pre-mRNAs); in addition, HERDS should also contain RNA of nucleolar origin, since remnants of the pre-ribosome fraction deriving from the nucleolar GC were sometimes found (Biggiogera *et al*. 1997b). In fact, it has been reported that all nuclear RNAs aggregate to rRNA in apoptotic nuclei (Halicka *et al*. 2000). The presence of rRNA (and nucleolar proteins) within HERDS has been indicated as a sign of "no-return" RNP segregation (Biggiogera *et al*. 2004).

Also the extruded RNP complexes are always associated with RNAs, as shown by ultrastructural cytochemistry (Biggiogera *et al*. 1998). RNA remnants are found also in apoptotic blebs (Biggiogera *et al*. 1998; Rosen and Casciola-Rosen 1999; Halicka *et al*. 2000), suggesting that RNA degradation in HERDS can also be incomplete. The release of RNA at the cell surface and the intracellular degradation by RNase activities (as it occurs for 28S rRNA: Delic *et al*. 1993) can account for the decrease in the amount of total RNA described in apoptotic cells.

Nadano and Sato (2000) proved that RNA degradation may be responsible for the inhibition of protein synthesis during apoptosis induced by death receptor engagement, and according to Degen *et al*. (2000) the mechanism of rRNA cleavage can be considered as a non-specific event reflecting the generalized shut-down of translation. The massive exit of nuclear RNAs from the nucleus into the cytoplasm of apoptotic cells (**Fig. 2A, 2B**) obviously results in an irreversible arrest of RNA maturation and the consequent blockade of protein synthesis; in parallel, the endonucleolytic degradation of DNA determines the arrest of nuclear transcription. These events have the adaptive role of inducing the interruption of two key functional processes (transcription and protein synthesis) in apoptotic cells (Pellicciari *et al*. 2005).

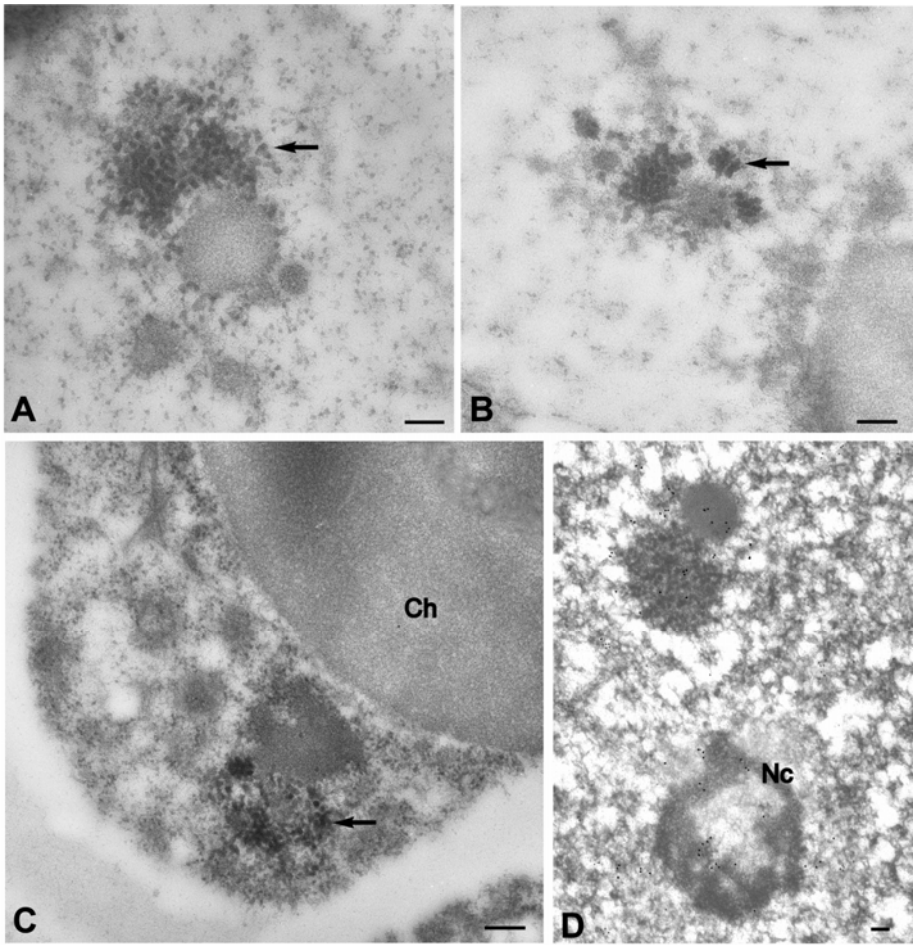

Fig. 2 HERDS formation and extrusion. (**A**) Apoptosis in thymocytes. Staining for RNP. HERDS are clearly heterogeneous, and often contain clusters of strictly packed peri-chromatin-like granules (arrow). (**B**) After specific EDTA staining for RNA with terbium, the presence of RNA inside the granules (arrow) is evident. (**C**) Etoposide treated thymocyte. HERDS are exported in the cytoplasm, and chromatin (Ch) segregate into condensed masses. PG-like structures (arrow) are still present. (**D**) ATP-depleted thymocyte after etoposide. Inside the nucleus, HERDS are present nearby a healthy nucleolus (Nc). Immunolabelling for the m3G-cap of snRNAs is present both on HERDS (recognizing U1, U2, U5, U4/6 snRNAs) and also on the nucleolus (where only U3 snRNA is present). Bar = 250 nm.

MIGRATION TO THE CYTOPLASM OF HERDS IS (AT LEAST IN PART) AN ACTIVE MECHANISM

After a blockade of ATP synthesis, cells can survive, although for a limited period, utilizing only the ATP reserve present within the cell (Shav-Tal *et al*. 2004) and can recover without a significant increase in the cell death rate (Cisterna *et al*. submitted). In order to better understand the mechanism related or directly responsible of HERDS formation and export, we induced apoptosis on isolated thymocytes by etoposide treatment, and then blocked ATP synthesis for 30 min before fixation and embedding for EM.

In ATP-depleted cells, in comparison with control cells, we have found a decrease in the number of cells displaying clear signs both of apoptosis and HERDS export toward the cytoplasm and, mainly, inside the blebs. This finding seems to imply that the energy-dependent processes present during apoptosis involve also the segregation of RNP structures.

The cells stimulated to undergo apoptosis, in fact, show patterns which clearly differ from those of ATP-depleted cells (**Fig. 2A-C**); the latter look more "healthy" and less frequent are the signs of RNP segregation in blebs or even karyorrhexis. The most striking feature is, however, the presence of an almost morphologically normal nucleolus inside the cell nucleus, often not far away from HERDS (**Fig. 3D**). HERDS formed in these conditions do not seem to contain immunodetectable nucleolar antigens, which support the hypothesis of the "no-return antigens" (Biggiogera *et al*. 2004): only when nucleolar proteins (or nucleolar components) are associated with HERDS in the final stages of cell death can this be achieved.

Given the diminished presence of HERDS inside blebs after ATP depletion, it seems reasonable to think that the migration of RNPs might be (at least in part) an energy-driven mechanism, in order to dispose of structures in a faster and safer way.

The diminished movements of HERDS, however, could also be linked to a cascade of events which are energy-dependent only in their first steps.

MIGRATION OF NUCLEAR PROTEINS INTO CYTOPLASMIC BLEBS MAY BE A NON-CHAOTIC PROCESS

In non apoptotic cells, the whole nuclear organization essentially depends on chromatin structuring which is responsible for the final nuclear architecture (Cremer and Cremer 2006a, 2006b). During apoptosis, the caspase-dependent activation of nucleases (Torriglia *et al*. 2005) results in a dramatic restructuring of the whole chromatin domains; as soon as the supporting structure (i.e. chromatin) is fragmented, the whole interchromatin super-domain where RNP-containing structures are located is also affected and collapses: already in early apoptosis, non-nucleolar RNPs are displaced from their original locations and associate with HERDS where nucleolar components may also be found. Following the partial disassembling of the nuclear envelope, RNP-containing aggregates as well as other non-RNP proteins can move to the cytoplasm, to be released within blebs.

Light and electron microscopy demonstrated that the cytoplasmic fragments released at the surface of apoptotic cells apparently differ in size, content and surface reactivity based on cytochemical techniques (Cline and Radic 2004) and may contain heterogeneous aggregates of nucleic acids (either DNA or RNA) and nuclear protein components (Halicka *et al*. 2000 and pers. obs.). In addition, there is a growing body of evidence that the massive migration of nuclear components to the cytoplasm might be a non-chaotic process: actually, large apoptotic bodies contain nuclear antigens (Rosen and Casciola-Rosen 1999) whereas small blebs preferentially contain molecules which associate with the endoplasmic reticulum and membrane scaffolding; also the RNA and DNA remnants are separated and extruded in different apoptotic bodies (Halicka *et al*. 2000 and pers. obs.).

A mechanism for these otherwise inexplicable events

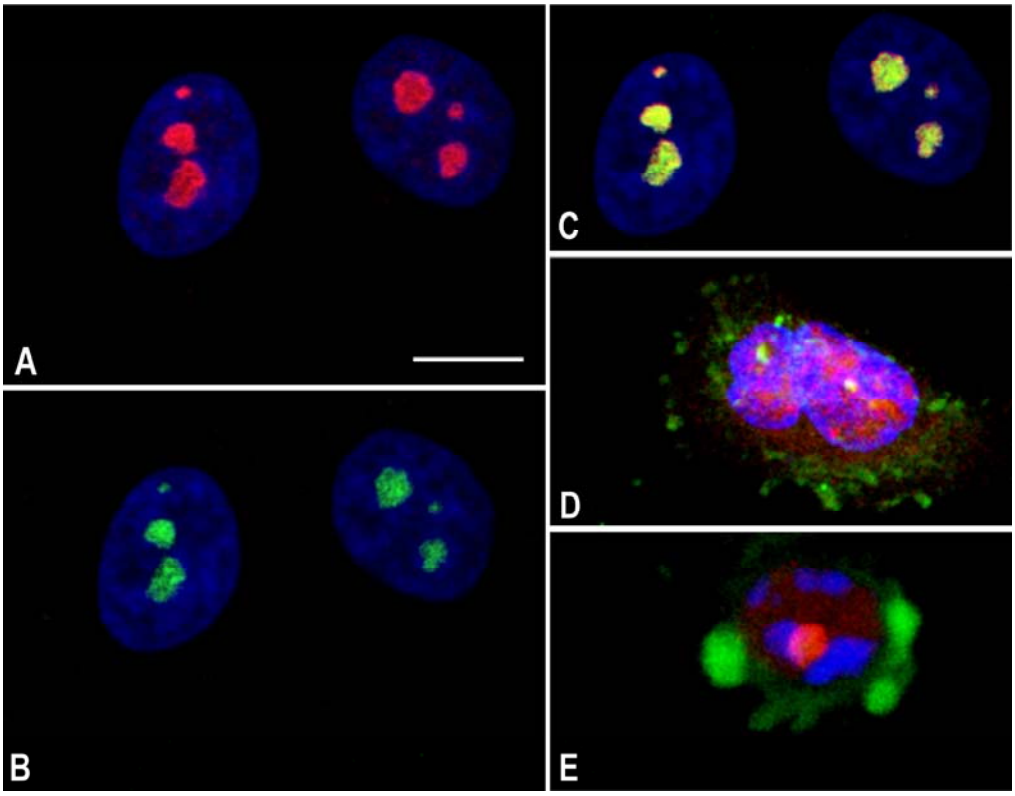

Fig. 3 (**A, B**) Dual immunolabeling for the phosphorylated c-Myc (a: red fluorescence) and fibrillarin (b: green fluorescence) in untreated control HeLa cells; (**C**) Merged image showing the co-localization of phosphorylated c-Myc and fibrillarin in the nucleoli of the control HeLa cells; (**D**) early apoptotic HeLa cell in an etoposide-treated culture demonstrating that fibrillarin first moves into the cytoplasm, whereas phosphorylated c-Myc does not locate in the nucleolus anymore, but is still present in the nucleus; (**E**) late karyorrhexic apoptotic HeLa cell in an etoposide-treated culture showing that fibrillarin locates in peripheral blebs, whereas phosphorylated c-Myc still aggregates in the center of the cytoplasm. Bar = 10 μm.

may possibly be found in a recent report by Soldani *et al*. (2006) who described the dynamic redistribution of three nucleolar proteins (Ki-67, fibrillarin, phosphorylated c-Myc) during apoptosis. In early apoptotic phases, the immunolabelings for Ki-67 and fibrillarin are similar as during interphase, whereas P-c-Myc starts moving from the nucleolus to the nucleoplasm, in the form of discrete immunopositive dots. In middle apoptosis, Ki-67 immunopositivity is lost (as a consequence of proteolytic cleavage) and fibrillarin is extruded into the cytoplasm, whereas P-c-Myc is still detectable in the interchromatin space as a diffuse signal. By late apoptosis, both fibrillarin and P-c-Myc are only found in the cytoplasm, but at different locations: P-c-Myc mainly form dense aggregates close to the nuclear fragments, whereas fibrillarin mainly locates in the peripheral cytoplasmic blebs (**Fig. 3**).

In apoptosis, the cell dies through an ordered and organized series of molecular events, the different steps being related to each other and often linked in a cause-effect relationship. The collapse of chromatin likely is the driving force disrupting the whole nuclear architecture: the molecular interactions which allow transcription and RNA maturation to take place in non-apoptotic nuclei are irreversibly altered and chromatin-bound or RNP-associated proteins first undergo relocation and rearrangement in the nucleus, and then eventually move to the cytoplasm, with a different kinetics. The cytoplasm also undergoes reorganization and degradation in apoptotic cells, the fragmentation of the whole vacuolar system (endoplasmic reticulum and Golgi) and the restructuring of the cytoskeleton components being relatively earlier events than nuclear disassembly.

This makes it likely that the heterogeneous content of apoptotic blebs might simply result from the presence of different cell components close to the cell surface in different moments: thus, cytoplasm components (from the vacuolar system and the membrane skeleton) would be first released inside small blebs, whereas nucleus-derived components would be preferentially located in larger apoptotic bodies which form in late apoptosis; their content may be variable depending on the dynamic of passage into the cytoplasm of the molecular aggregates of nuclear origin, so that e.g. fibrillarin is found in blebs earlier than phosphorylated c-Myc. This would also explain why the segregation of nuclear components observed in early apoptotic nuclei before karyorrhexis (reviewed in Biggiogera *et al*. 2004; Pellicciari *et al*. 2005) may be preserved even in the cytoplasm of apoptotic cells resulting in the final formation of blebs with different contents.

CONCLUDING REMARKS

The final step of the apoptotic process is phagocytosis: this usually results in the safe and rapid removal of apoptotic cells which remain in the tissues for relatively short periods. Because of this, developing lymphocytes (which reside in organs where clearance mechanisms are highly effective) will usually not encounter late apoptotic products and tolerance towards late apoptotically modified molecules cannot be established (Cline and Radic 2004). However, if phagocytosis is defective or when apoptosis is so massive to overwhelm the clearance capability of scavenger cells, the disposal of apoptotic cells may become insufficient and late apoptotic cells accumulate in the tissue where they can be phagocytosed by antigen-presenting cells and becoming visible to the immune system eventually eliciting an autoimmune response (reviewed in Rovere-Querini *et al*. 2005; Mahoney and Rosen 2005). In fact, autoimmunity requires an appropriate genetic background to take place and can be explained non only by the failure to eliminate auto-reactive cells (Cline and Radic 2004; Kaplan 2004) but also by a *correct* immune response against auto-antigens derived from chemically modified cellular components (Rosen and Casciola-Rosen 1999; Mahoney and Rosen 2005): HERDS may actually be considered as *new* molecular aggregates since they contain heterogeneous complexes of RNA, RNPs and other nuclear proteins modified by a definitely partial proteolytic cleavage, as demonstrated by the preserved immunolabeling capability even inside apoptotic blebs.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. T. E. Martin for the anti-hnRNP core proteins antibody. This work was supported by the University of Pavia (Fondo di Ateneo per la Ricerca 2006-2007) and MIUR, PRIN Project 2005058254.

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