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PROSPECT

Nuclear Apoptotic Changes: An Overview

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Abstract Apoptosis is a form of active cell death essential for morphogenesis, development, differentiation, and homeostasis of multicellular organisms. The activation of genetically controlled specific pathways that are highly conserved during evolution results in the characteristic morphological features of apoptosis that are mainly evident in the nucleus. These include chromatin condensation, nuclear shrinkage, and the formation of apoptotic bodies. The morphological changes are the result of molecular alterations, such as DNA and RNA cleavage, post-translational modifications of nuclear proteins, and proteolysis of several polypeptides residing in the nucleus. During the last five years our understanding of the process of apoptosis has dramatically increased. However, the mechanisms that lead to apoptotic changes in the nucleus have been only partially clarified. Here, we shall review the most recent findings that may explain why the nucleus displays these striking modifications. Moreover, we shall take into consideration the emerging evidence about apoptotic events as a trigger for the generation of autoantibodies to nuclear components. J. Cell. Biochem. 82: 634–646, 2001.

Key words: apoptosis; chromatin condensation; proteolysis; autoantibodies

Apoptosis is a form of regulated cell death which results in the orderly removal of cells that are senescent, unneeded or defective and destined to die. Apoptosis is also complementary to mitosis, and these two phenomena determine maintenance, growth or involution of tissues. Therefore, apoptosis plays a major role during development, maturation of the immune system, and aging. Furthermore, while excessive apoptosis results in some neurodegenerative disorders due to cell loss, defects in apoptosis promote tumorigenesis by prolonging cell life span and hence cell accumulation [Hengartner, 2000]. Because apoptosis is also required for tumor eradication by the immune system, anticancer drugs, and irradiation, cancer-associated defects in the cellular apoptotic machinery play a fundamental role in therapeutic treatment failures [Reed, 2000]. Apoptotic cell death was first described by Kerr et al. [1972] almost 30 years ago and was distinguished from necrosis exclusively on the basis of morphological criteria such as chromatin condensation and the formation of apoptotic bodies. Subsequently, it has become clear that apoptosis is also characterized by a variety of biochemical changes, which occur in several organelles, including the nucleus. Albeit the precise role of nuclear apoptotic changes has been questioned [Robertson et al., 2000], there is no doubt that the events taking place in the nucleus are striking, and some of them (e.g. DNA degradation and proteolysis of selected polypeptides) are routinely used as biochemical markers of apoptosis. Even though in recent years the interest in apoptotic nuclear modifications has considerably shifted from morphological changes to proteolytic activation, a considerable emphasis continues to focus on chromatin

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condensation, nuclease activation, DNA cleavage, and nuclear fragmentation. However, many of the biochemical events and molecular mechanisms underlying apoptotic morphological changes have been hard to define, mostly because apoptosis often occurs in an asynchronous way.

This review focuses on the most recent advances of our knowledge about both morphological and biochemical nuclear events characteristic of apoptotic cell death and should be intended as an updating of other comprehensive articles dealing with this issue [Earnshaw, 1995; Martelli et al., 1997; Robertson et al., 2000].

THE APOPTOTIC NUCLEAR MORPHOLOGICAL CHANGES

The morphological changes typical of apoptosis are well known: they consist of a collapse of chromatin (which, in many cases adopts a striking crescent or "half-moon" shape) against the nuclear periphery, a progressive condensation of chromatin, a shrinkage of the entire nucleus into a single ball or, in other cases, chromatin budding outward into smaller balls resembling a cluster of grapes, with each grape being surrounded by nuclear envelope [Earnshaw, 1995]. These balls are referred to as apoptotic bodies and some of them contain only DNA whereas others contain only RNA [Halicka et al., 2000]. The nuclear envelope remains morphologically intact even though the nuclear pores redistribute by sliding away from the surface of the condensed chromatin domains and accumulating between them [Earnshaw, 1995]. The apoptotic nuclear changes are conceivably due to both DNA fragmentation and proteolysis of key nuclear polypeptides. The endonuclease activities, producing both large and small DNA fragments, suggested to be involved in apoptosis are quite numerous and their detailed reviewing is beyond the scopes of this article. At present, increasing interest surrounds caspase-activated DNase (CAD), an enzyme which cleaves DNA at internucleosomal spaces via an interaction with both histone H1, high-mobility group (HMG) protein 2, and topoisomerase IIα [Counis and Torriglia, 2000; Durrieu et al., 2000; Robertson et al., 2000]. Regarding proteases, a family of cysteine-aspartate enzymes (referred to as caspases) are of critical importance in apoptosis [Earnshaw et al., 1999, and references therein]. Recent data have highlighted that two main initiator caspases (-8 and -9) are able to signal via distinct pathways and this ultimately results in the recruitment and activation of effector caspase -3 and -6, that are both capable of cleaving numerous nuclear proteins [Robertson et al., 2000]. Caspases are present as inactive enzymes (pro-caspases) in the cytoplasm. Therefore, an issue of fundamental importance is their identification within the nucleus. A few reports have hinted at the presence of both procaspases and caspases -1 and -2 in the nuclear compartment [Robertson et al., 2000]. So far, however, no nuclear substrates for these proteases have been discovered. Evidence is being accumulated that also noncaspase proteases, including cathepsins, calpains, granzymes, and the proteasome complex, have roles in mediating cell death [Johnson, 2000]. A caspase-dependent nuclear translocation of granzyme B has been reported in Jurkat T cells [Pinkoski et al., 2000]. At present, no nuclear substrates for proteases other than caspases have clearly been identified, even though apoptotic cleavage of the abundant 240-kDa nuclear matrix protein NuMA (for nucleus-mitotic apparatus) was delayed by the chymotrypsin inhibitor, TPCK, but not by the caspase inhibitor, Ac-YVAD-cmk [Gueth-Hallonet et al., 1997]. However, others have reported that several recombinant caspases (-3, -4, -6, -7, -8) cleave, at least in vitro, NuMA [Hirata et al., 1998]. Our more recent results also suggest an involvement of caspase activity in the NuMA cleavage during apoptosis of HL-60 cells [Bortul et al., 2001].

In addition to the more established changes, over the last few years it has become clear that other morphological modifications occur in the apoptotic nucleus. Several independent investigators [Biggiogera and Pellicciari, 2000] have demonstrated that during apoptosis the nucleus contains segregated clusters of ribonucleoprotein (RNP) referred to as HERDS (for heterogeneous ectopic RNP-derived structures). We have found that HERDS are enriched in nuclear matrix proteins [Zweyer et al., 1997]. It should be emphasized, however, that HERDS are not exclusively associated with apoptosis, because they are likely to reflect a transcriptional block [Biggiogera and Pellicciari, 2000] and are seen for example also during terminal differentiation of mammalian erythroblasts, in parallel

with chromatin condensation [Biggiogera et al., 1999].

In contrast, peculiar to apoptosis seem to be the very early changes (preceding the externalization of phosphatidylserine at the plasma membrane level, chromatin condensation, and DNA fragmentation) described by Johnson et al. [2000] in staurosporine- or etoposide-treated Jurkat T cells. They consist of heavily convoluted nuclei exhibiting numerous cavitations plus dilation of the nuclear envelope. An example of these ultrastructural modifications is presented in Figure 1C. Interestingly, these changes do not require the activity of effector caspase-2, -3, or -7, but are probably dependent on some member of group III caspases (-6, -8, -9, -10), most likely caspase-9.

Nuclear Domains and Apoptosis

A wealth of evidence, collected over the past fifteen years, has convincingly shown the nucleus to be a highly organized organelle, which contains not only its own skeleton but also distinct suborganelles, or domains, where different functions take place with speed, efficiency, and unrivaled fidelity [Lamond and Earnshaw, 1998]. In the following sections, we shall briefly illustrate the most recently gained information about changes that affect some of the nuclear domains during apoptosis.

1. The Nuclear Matrix

The insoluble nuclear skeleton is often referred to as the nuclear matrix or scaffold, a dynamic structure, which resists treatment of isolated nuclei with detergents, solutions of high ionic strength, and nucleases. The matrix is mainly composed of nonhistone proteins and RNA and forms an elaborate anastomosing three-dimensional (3D) fibrogranular network through the nucleus, intersecting with the peripheral lamina [Pederson, 2000]. It is prominently involved in organizing 30 kb chromatin loops into functional domains through intrinsic interactions with nucleic acids. To this end, the genome possess A-T rich DNA sequences, termed matrix/scaffold attachment regions (M/SARs), that are implied in a specific anchorage of chromatin loops to the nucleoskeleton [Boulikas, 1995]. Although an early report ruled out any involvement of the nuclear matrix during glucocorticoid-induced apoptosis in thymocytes [Arends et al., 1990], there is now clear indication that the nucleoskeleton is markedly

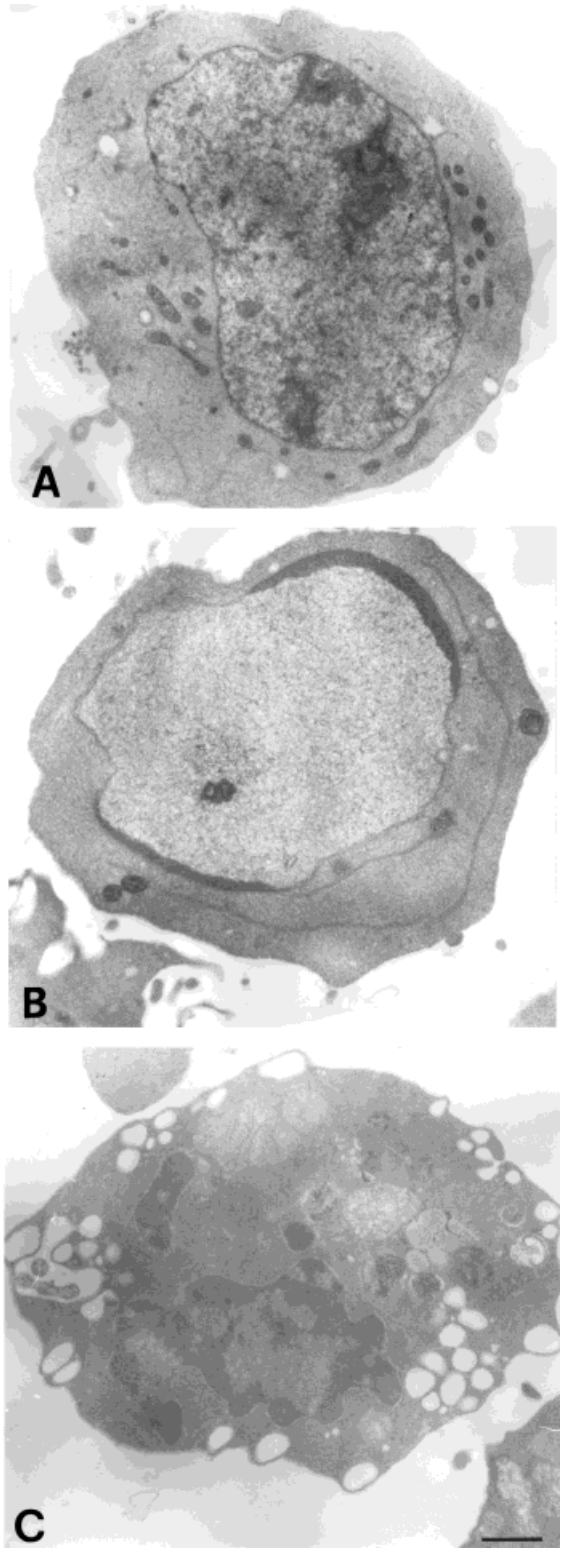

Fig. 1. Transmission electron microscopy analysis of staurosporine-treated Jurkat T lymphocytes. **A**: untreated cell. **B**: cell treated for 3 h with 0.5 μM staurosporine. **C**: cell treated for 3 h with 5 μM staurosporine plus 100 μM of Z-DEVD-FMK (an inhibitor of caspase-3). Johnson et al. [2000]. Scale bar: 1 μm.

affected during apoptotic cell death. The evidence which supports this involvement comes from both morphological and biochemical investigations. Our laboratory has shown that marked ultrastructural changes are detectable in the nuclear matrix fraction prepared from apoptotic HL-60 cells. These changes are always detectable, independently of the methods employed to isolate the matrix [Martelli et al., 1999a,b]. We, and others, also reported, by means of immunofluorescence analysis, striking modifications in the spatial distribution of NuMA [Sodja et al., 1998; Martelli et al., 1999a], a further indication of the changes occurring at the nuclear matrix level. As far as biochemical alterations are concerned, the current opinion holds that early proteolysis of key nuclear matrix proteins may open sites of nuclease hypersensitivity similar to those observed in transcriptionally active regions of chromatin [Krystosek, 1999], given that both protease and endonuclease activities are required for DNA fragmentation in apoptotic cells. Moreover, proteolysis probably facilitates the breakdown of the nuclear matrix itself and, ultimately, of the entire nucleus [Robertson et al., 2000].

Consistently with this model, some M/SAR-binding proteins are cleaved during apoptosis. These include topoisomerase IIα [Casiano et al., 1996], NuMA [Gueth-Hallonet et al., 1997], SAF-A [Göhring et al., 1997; Kipp et al., 2000], lamin B1 [Dynlacht et al., 1999], lamins A and C [Takahashi et al., 1996], and SATB1 [Gotzmann et al., 2000]. It is of great interest that, in the case of SAF-A (a protein which can bind both DNA and RNA), the cleavage occurs within the bipartite DNA-binding domain, resulting in loss of DNA-binding activity and a concomitant release of the protein from the nuclear matrix [Göhring et al., 1997]. In contrast, cleavage of SAF-A did not affect the association of the protein with heterogeneous nuclear RNP complexes. This indicates that the function of SAF-A in RNA metabolism is not compromised in apoptosis whereas its cleavage may contribute to apoptotic nuclear breakdown. The behavior of SAF-A differs from that of SATB1, because in latter case the cleavage left a C-terminal fragment still possessing both the proposed M/SAR-binding domain and the homeodomain [Gotzmann et al., 2000], so that the cleaved protein still associates with the nuclear matrix.

Although the morphological patterns of apoptotic nuclear changes are quite similar even in different experimental models [e.g. Falcieri et al., 2000], recent evidence supports the notion that, depending on the inducing agent, the hierarchical sequence and kinetics of degradative events (both proteolysis and DNA fragmentation) contributing to nuclear disassembly during apoptosis are variable [Stolzenberg et al., 2000]. For example, a recent study has shown that in heat-induced apoptosis of HL-60 cells, cleavage of lamin B and NuMA occurred much sooner than when other inducers (radiation or etoposide) were employed [Dynlacht et al., 2000]. It has been proposed that the differences may depend on the activated signaling pathways being both cell type-specific and highly dependent on the initiating stimulus. These findings could also explain the divergent results obtained by some investigators when studying the proteolysis of the same M/SAR proteins in different apoptotic models [Göhring et al., 1997; Martelli et al., 1999a; Gotzmann et al., 2000]. Moreover, the results by Dynlacht et al. [2000] supports an emerging paradigm of apoptosis which suggests a hierarchy of proteolytic events in which cleavage involves the stepwise activation of multiple caspases with sequence and substrate specificity [Johnson et al., 2000]. Indeed, proteolysis of NuMA and lamin B1 involves at least two different proteases and the massive degradation of these S/MAR proteins occurs either concomitantly or after DNA fragmentation. Thus, it was speculated that DNA fragmentation is not dependent on a large-scale degradation of proteins, but rather DNA degradation is a prerequisite for further proteolysis.

Apart from the specific cleavage of S/MAR proteins, other reports have highlighted the presence, in the apoptotic nuclear matrix, of qualitative changes in the polypeptide profile as observed by means of two-dimensional (2D) polyacrylamide gel electrophoretic separations [Gerner et al., 1998; Martelli et al., 1999a,b]. Gerner et al. [1998] have hypothesized that these new proteins (presumably translocating from cytoplasm to the nucleus) are proteases, DNases, transcription factors, and protein kinases involved in the apoptotic nuclear destruction. A protein kinase which, in human prostate adenocarcinoma cell lines, translocates from the cytoplasm to the nucleus in response to apoptotic agents such as etoposide

and diethylstilbestrol, is casein kinase 2 (CK2) [Guo et al., 2001]. The activity of CK2 is generally elevated in rapidly proliferating tissues and the nuclear matrix is an important subnuclear domain for its functional activity. The shuttling of CK2 to the nuclear matrix may reflect a protective response to chemical- (but not receptor-) mediated apoptosis. Therefore, the destruction of the nuclear matrix during apoptosis may also serve for destroying a nuclear milieu important for cell survival.

2. The Nucleolus

This organelle has been considered for decades the ribosome factory and the site for ribosomal RNA synthesis and processing [Sheer and Hock, 1999]. However, this is a reductive view, as recent studies have highlighted that the nucleolus participates in many other aspects of gene expression [Pederson, 1998]. Nucleolar segregation (i.e. the separation and condensation of the granular and dense fibrillar nucleolar components) is seen in some types of apoptosis but, on the whole, the nucleolus is generally regarded as a nuclear domain very resistant to apoptosis [Columbaro et al., 1999; Martelli et al., 1999b]. Thus, in some apoptotic models, such as thymocytes or other lymphoid cells, nucleolar components can be recognized for a long time. Even by means of conventional ultrastructural staining, granular and fibrillar components can be identified, closely associated with each other or scattered throughout the cytosol. However, in other cell lineages undergoing apoptosis, nucleoli seem to progressively disappear and only rare nucleolar structures can be revealed in late apoptotic stages [Martelli et al., 2000a]. We have found that some nucleolar proteins (C23/nucleolin, B23/nucleophosmin, fibrillarin) undergo a redistribution in their spatial distribution in camptothecin-treated HL-60 cells [Martelli et al., 2000a]. A shift in its location from nucleolus to nucleoplasm had been previously reported for B23/nucleophosmin also in apoptotic K562 and HeLa cells [Chan and Chan, 1999]. Moreover, we have demonstrated that HERDS originate at least in part from the nucleolus [Zweyer et al., 1997], given that they also contain NOR-90/UBF (upstream binding factor), the main regulatory element of rDNA transcription [Kuhn and Grummt, 1992]. At variance with C23/nucleolin and B23/nucleophosmin, UBF is cleaved during apoptosis [Casiano et al., 1996; Martelli et al., 2000a]. This is in agreement with an immunostaining investigation which has shown that UBF was absent or clearly diminished in several cases of Hodgkin's disease, where apoptosis is known to take place [Torres-Montaner et al., 2000]. Interestingly, UBF expression was markedly reduced in Reed-Sternberg and Hodgkin cells that are highly diagnostic for this type of malignant lymphoproliferative disorder. Therefore, it was concluded that this type of immunostaining may prove valuable for identification of early stages of apoptosis in cytological and histopathological specimens. Apoptotic UBF cleavage might be in relationship with the findings by Stegh et al. [1998], who described a novel 37-kDa protein, DEDD, situated on the CD95 (Fas /APO-1) signaling pathway. Upon Fas stimulation, DEDD translocates to the nucleolus in a manner, which is partly caspase-dependent. In the nucleolus, DEDD colocalizes with UBF (so that it may facilitate UBF cleavage) and, in a reconstituted in vitro system, DEDD inhibits rDNA transcription.

It should also be reminded that morphological and histochemical evidence has determined that some of the electron dense material present in proximity of the nuclear envelope of apoptotic cells (previously thought to represent chromatin) is the result of the segregation of nucleoli in association with the nuclear membrane, while some DNA migrates into the bulk of nucleoli at the early stages of cell death in the pluripotent murine hematopoietic stem cell line FDCP-mix [Reipert et al., 1999]. In light of these findings, it has been proposed that the nucleolar segregation might be the overriding process, which is responsible for the overall apoptotic morphology of the nucleus.

3. The Nuclear Periphery

Apart from the degradation of the nuclear lamina [e.g. Oberhammer et al., 1994], other peripheral nuclear domains are affected by the apoptotic process. The caspase-dependent cleavage of several nuclear membrane and nuclear pore complex proteins has been described. These include the lamin B receptor (LBR), lamin-associated polypeptide (LAP) 2α, nucleoporin (Nup) 153 and 270, as well as RanGAP1 [Duband-Goulet et al., 1998; Buendia et al., 1999; Faleiro and Lazebnik, 2000; Gotzmann et al., 2000]. Cleavage of LBR, LAP2α and Nup 153, that are exposed at the inner face of the

nuclear envelope and all interact with chromatin, suggests that their protelysis conceivably allows the detachment of the nuclear envelope from chromatin. Accordingly, the LAP2α cleavage site is localized to its chromosome-binding domain [Gotzmann et al., 2000]. Furthermore, it is likely that another consequence of these proteins been cleaved is the clustering of nuclear pore complexes in the plane of the membrane, a consistent feature of apoptosis [Falcieri et al., 1994; Reipert et al., 1996]. RanGap1 is a soluble component of the nuclear transport machinery [Cole and Hammell, 1998; Moroianu, 1999] while Nup 270 is a constituent of the nuclear pore complex [Stoffler et al., 1999; Ryan and Wente, 2000]. Caspase-9, which is activated earlier than caspase-3, was found to activate directly or indirectly nuclear transport and to increase the diffusion limit of nuclear pores. This increase then allows caspase-3, which is usually cytoplasmic, to gain access to its nuclear targets [Faleiro and Lazebnik, 2000]. Cleavage of RanGP1 and Nup 270 conceivably contributes to modifications in nuclear pore permeability, even though, obviously, the possibility exists that several other nuclear pore components are caspase substrates and their degradation causes an increase in nuclear permeability. In fact, more than 50 proteins of the vertebrate nuclear pore have so far been identified [Stoffler et al., 1999; Ryan and Wente, 2000].

Post-Translational Modifications of Nuclear Proteins During Apoptosis

Post-translational modifications of proteins (most notably phosphorylation) have long been recognized as a way to finely tune protein structure and function also in the nucleus. For example, phosphorylation state of histones is known to deeply influence chromatin structure [Ajiro and Nishimoto, 1985], while nuclear envelope breakdown, the process by which the physical barrier between cytoplasm and nucleus is dissolved to allow for cell division, requires the involvement of multiple mitotic lamin kinases, including p34cdc2/cyclin B kinase and protein kinase C (PKC) [Fields and Thompson, 1995]. There is now evidence that phosphorylation of nuclear proteins occurs during apoptosis. Independent groups have reported that histone H3 [Waring et al., 1997], H2B and H4 [Ajiro, 2000], and H2AX [Rogakou et al., 2000] are all hyperphosphorylated in apoptotic cells. Hyperphosphorylation of HMGA1a protein has been shown to take place during the early stages of apoptosis in various hematopoietic cell lines [Diana et al., 2001]. The hyperphosphorylation of chromatin-bound, replication A middle subunit (RPA32) has been reported in apoptotic Jurkat T lymphocytes [Treuner et al., 1999]. Hyperphosphorylation of lamins during apoptosis has also been described in HL-60 cells [Shimizu et al., 1998].

An issue of fundamental importance is the identification of the protein kinases that are responsible for the hyperphosphorylation of the above-listed substrates. Here, our knowledge, although limited, is in rapid expansion. Apoptotic histone H3 phosphorylation was prevented by genistein, an inhibitor of protein kinase A (PKA) [Waring et al., 1997], while PKC has been proposed to be involved in the phosphorylation of both histone H2B and lamin B [Shimizu et al., 1998; Martelli et al., 1999c; Ajiro, 2000; Cross et al., 2000 for a comprehensive review on nuclear PKC and apoptosis]. Among the various PKC isoforms, the -δ isozyme is now attracting considerable attention since it has been shown to translocate to the nucleus early following apoptotic stimuli [Scheel-Toellner et al., 1999; Cross et al., 2000]. Once in the nucleus, the full-length PKC-δ is cleaved by caspase-3 which generates a constitutively active catalytic fragment. If human T cells, deprived of growth factors, are exposed to interferon-β (a cytokine with a rescuing effect) the proteolytic cleavage of nuclear PKC-δ is inhibited [Scheel-Toellner et al., 1999]. Moreover, in HL-60 cells treated with ara-C, the PKC-δ specific inhibitor, Rottlerin, inhibited lamin phosphorylation and proteolysis. In contrast, either an inhibitor of PKC-α, -β, and-γ (Go6976) or an inhibitor of p34cdc2 (Olomoucine) were unable to block both phosphorylation and proteolysis of lamin B [Cross et al., 2000]. The findings about nuclear protein phosphorylation during apoptosis are summarized in Table I.

There are also results suggesting the activation at the nuclear level of phosphatase activities during apoptotic cell death. HMGA1a is dephosphorylated in the highly condensed chromatin of apoptotic bodies [Diana et al., 2001] and an early dephoshorylation of some histone H1 subtypes, preceding DNA fragmentation in HL-60 cells treated with an inhibitor of topoisomerase I, has been described [Kratzmeier et al., 2000].

TABLE I. Nuclear Proteins Known to be Phosphorylated During Apoptosis

Protein	Cell type(s)	Phosphorylated residues	Involved kinase(s)	References
NuMA	Rat thymocytes	n.d.	n.d.	Weaver et al., 1996
Lamin B	HL-60 cells	n.d.	PKC-α	Shimizu et al., 1998
		n.d.	PKC-δ	Cross et al., 2000
RPA32	Jurkat T cells	n.d.	DNA protein kinase, cyclin-dependent kinases	Treuner et al., 1999
Histone H3	Mouse thymocytes, P815 cells	Serine-10	PKA	Waring et al., 1997
Histone H2B	Rat thymocytes, HL-60, HeLa, FM3A, N18 cells	Serine-32	n.d.	Ajiro, 2000
Histone H2AX	HL-60, Jurkat, MCF7 cells	Serine-139	n.d.	Rogakou et al., 2000
HMGA1a	HL-60, K562, NB4, U937 cells	n.d.	n.d.	Diana et al., 2001

n.d. : not determined.

As far as other post-translational modifications are concerned, histone H1 undergoes poly(ADP-ribosyl)ation during apoptosis [Yoon et al., 1996], a result which is consistent with the transient burst of poly(ADP-ribosyl)ation which occurs early during apoptosis in human osteosarcoma cells [Simbulan-Rosenthal et al., 1998]. Furthermore, apoptosis induces other protein modifications such as deacetylation and methylation [Utz and Anderson, 1998; Diana et al., 2001].

What may be the physiological relevance of all these apoptotic post-translational modifications of nuclear proteins? They have generally been interpreted as if they would either render chromatin more susceptible to endonuclease action and/or cause chromatin itself to condense, or facilitate the disassembly of the peripheral nuclear lamina. It has also been proposed that hyperphosphorylation of proteins during apoptosis may cause them to become more prone to proteolytic cleavage, as in case of NuMA [Weaver et al., 1996]. So far, however, a conclusive demonstration of the exact role(s) played by the apoptotic post-translational modifications of nuclear proteins is lacking. Nevertheless, as we shall see later, these modifications might have deep consequences on the autoantigenicity of the proteins.

Generation of Nuclear Lipid Second Messengers During Apoptosis

There is now compelling evidence that lipid second messengers are generated within the nucleus in response to a variety of proliferating and differentiating stimuli, and also during apoptosis [Martelli et al., 1999d; Cocco et al., 2001]. What may be the targets of intranuclear lipid second messengers? The most obvious are represented by the PKC isoforms that are present in the nucleus or can translocate there during apoptosis [Martelli et al., 1999c]. In this connection, we have shown that, in camptothecin-treated HL-60 cells, there is an early and transient rise in nuclear diacylglycerol (DAG) levels which precedes activation of PKC-α in the nuclear compartment. The use of specific pharmacological inhibitors led us to conclude that this DAG derived through the action of a nuclear phospholipase D (PLD). Propranolol, an inhibitor of PLD-dependent DAG generation, significantly reduced the number of HL-60 cells that underwent apoptosis following exposure to camptothecin [Martelli et al., 1999e]. In the erythro-myeloblastic cell line TF-1-33, ionizing radiations caused activation of neutral sphingomyelinase and ceramide generation only in highly purified preparations of nuclei [Jaffrézou et al., 2001]. Ceramide generation might be related to activation of PKC-ζ, which translocates to the nucleus in some apoptotic models [Martelli et al., 1999c]. However, Jaffrézou et al. [2001] also speculated about a possible involvement of ceramide in endonuclease and protease activation.

Apoptotic Versus Necrotic Nuclear Changes

As stated above, apoptosis was initially distinguished from necrosis on the basis of morphological criteria. Necrotic cells swell and loose the protective function of the plasma membrane, concomitant with anucleolytic pyknosis. Nevertheless, there is increasing awareness that apoptosis and necrosis represent only the extreme ends of a wide range of possible morphological and biochemical deaths. They can occur at the same time in tissues or cell cultures exposed to the same noxious agent and,

in many cases, it is the intensity of the same initial insult which decides the prevalence of either apoptosis or necrosis [Nicotera et al., 1999; Formigli et al., 2000]. As such, it is now apparent that the differences between necrosis and apoptosis might be less numerous than initially believed. Indeed, some of the biochemical changes that take place during apoptosis, such as proteolysis, have been reported to occur also during necrosis [Dynlacht et al., 1999; Bortul et al., 2001 and references therein]. We recently moved to analyze necrotic nuclear changes and to compare them to those occurring during apoptosis, utilizing HL-60 cells [Bortul et al., 2001]. Ultrastructural observations revealed that slight morphological changes were present in the nuclear matrix fraction prepared from necrotic cells. However, these modifications (mainly consisting of a rarefaction of the inner fibrogranular network) were not as striking as those we have previously described in apoptotic HL-60 cells [Martelli et al., 1999a,b]. By means of immunofluorescence staining, we demonstrated that the patterns given by antibodies directed against some nuclear proteins (lamin B1, NuMA, topoisomerase IIα, SC-35, B23/nucleophosmin) changed in necrotic cells. Although the changes in the spatial distribution of NuMA strongly resembled those described to occur during apoptosis, the modifications involving the localization of topoisomerase IIα (which concentreated in large and bright masses apparently contained within nucleoli) was peculiar to necrotic cells (Fig. 2). By immunoblotting analysis, we observed that some nuclear matrix proteins (SAF-A, SATB1, NuMA) were cleaved during necrosis, and, in the case of SATB1, the apoptotic signature fragment of 70-kDa was also present to the same extent in necrotic samples. Caspase inhibitors did not prevent proteolytic cleavage of the aforementioned polypeptides during necrosis, while they were effective if apoptosis was induced. In contrast, lamin B1 and topoisomerase IIα were uncleaved in necrotic cells, whereas they were proteolyzed during apoptosis [Bortul et al., 2001]. The specific cleavage of some nuclear proteins during necrosis had previously been demonstrated by Casiano et al. [1998]. It should also be mentioned that according to Dynlacht et al. [1999] lamin B was cleaved in radiation-exposed, necrotic HL-60 cells, albeit the cleavage pattern differed from that observed in apoptotic samples. Therefore, our findings have identified additional morphological and biochemical criteria that could be used to discriminate between the two types of cell death.

The Release of Sequestered Nuclear Antigens During Apoptosis: a Trigger of Autoimmunity?

The generation of autoantibodies to normally sequestered antigens, such as proteins residing in the nucleus, has puzzled immunologists for decades. These autoantibodies occur in a wide variety of pathological conditions, and are one of the hallmarks of a series of diseases that are referred to as "autoimmune". In the past, several hypotheses have been proposed to explain such a phenomenon, but none of them appeared really convincing or was supported by strong experimental evidence [Rodenburg et al., 2000]. A rapidly emerging view is that production of autoantibodies might be induced by repeated exposure to the immune system of excessive amounts of intracellular material released from dying cells [Rosen and Casciola-Rosen, 1999]. In other words, the cells dying by apoptosis are considered to be the supplier of autoantigens and there are now several noteworthy clues that support such a model. Probably the strongest evidence comes from the observation that many proteins that are autoantigenic in patients suffering from autoimmune diseases are modified in an unique way during apoptosis. As a consequence, cryptic/novel epitopes may be uncovered/created, so

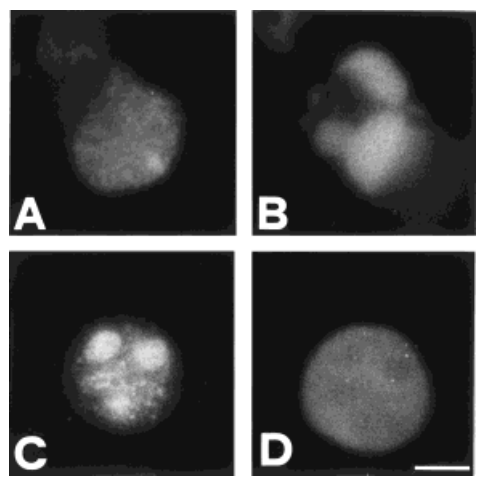

Fig. 2. Immunofluorescence staining of apoptotic (**A**) and necrotic (**C**) HL-60 cells reacted with an anti-topoisomerase IIα monoclonal antibody. (**B**) and (**D**) show the same cell stained for DNA with 0.01μg/ml DAPI (4′-6-diamidino-2-phenylindole). Bortul et al. [2001]. Scale bar: 5 μm.

that no tolerance exists and, in susceptible individuals, an autoimmune response may ensue. For example, impaired phagocytosis of apoptotic bodies, as observed in systemic lupus erythematosus patients, might alter the clearance of apoptotic cell debris resulting in an increased exposure to the immune system of modified self-components [Rosen and Casciola-Rosen, 1999].

Clearly, the best characterized modification of autoantigenic proteins which occurs during apoptosis is represented by their specific cleavage, as outlined above. Autoantibodies from patients suffering from autoimmune diseases are frequently directed to nuclear proteins that are cleaved during apoptosis and they recognize also the cleaved fragments [Casiano et al., 1996]. Another modification which may affect the antigenicity of nuclear proteins is phosphorylation/dephosphorylation. In addition to the examples of phosphorylation reported above, it is important to mention here that autoimmune sera from patients suffering from systemic lupus erythematosus selectively immunoprecipitated from apoptotic lysates four phosphorylated splicing factors (pp54, pp42, pp34, and pp23) associated with the U1 small ribonucleoprotein (snRNP) [Utz et al., 1997]. Moreover, several nuclear autoantigens undergo a striking redistribution and concentration during apoptosis. Apoptotic bodies are highly enriched in snRNPs, Ku, and poly(ADP-ribose)polymerase [Rosen and Casciola-Rosen, 1999]. The same holds true for HERDS, as these structures contain still immunodetectable snRNPs [Biggiogera et al., 1997a,b], nuclear matrix proteins [Zweyer et al., 1997], and RNA [Biggiogera et al., 1998]. Moreover, C23/nucleolin and B23/nucleophosmin remain detectable in granular material present amid micronuclei of late apoptotic cells [Martelli et al., 2000a], while fibrillarin is present in nucleolus-like bodies, that resemble functional nucleoli and are observed inside the cytoplasmic fragments blebbing out at the cell surface during apoptosis induced by hypertonic stress [Pellicciari et al., 2000]. All of these different kinds of particles might be captured by the appropriate MHC class II molecules of antigen presenting cells so that previously cryptic peptides would elicit an antibody response.

As a first step towards the demonstration that inappropriate apoptosis might be related to the generation of antinuclear autoantibodies, we have shown that nuclear matrix proteins (for example, NuMA) are released from leukocytes dying of apoptosis in the supernatant of red blood cell units. By immunoblotting analysis and immunofluorescent staining, we have found that in 4 out of 38 multitransfused patients, autoantibodies against nuclear matrix proteins were present without any clinical or laboratory sign of autoimmune disease. One of the sera from these patients, recognizing a 64-kDa nuclear matrix protein, immunostained nuclear dots that were identified as coiled bodies because of their colocalization with p-80 coilin [Martelli et al., 2000b]. These results suggest that a massive amount of nuclear protein released during apoptosis, once repeatedly transfused, may induce an autoimmune response.

Also the presence of nucleic acids in apoptotic bodies should be considered important for a possible generation of autoantibodies. Indeed, systemic autoimmune disorders, such as lupus erythematosus, are often characterized by the presence of autoantibodies directed to nucleic acid-protein complexes present in the cell nucleus [Degen et al., 2000a]. Recent findings show that nucleic acids (not only DNA but also selected RNAs, such as the U1 snRNA molecule) are modified (i.e. cleaved by caspase-dependent nucleases) during apoptosis [Degen et al., 2000b]. Thus, these modifications may lead to autoantibody formation.

Concluding Remarks

Over the last few years our knowledge about nuclear apoptotic changes has improved in a substantial way. However, there are still many issues that need to be clarified to completely unravel the multiple and complex key steps involved in the execution phase of apoptosis. It is clear that caspase activation and DNA fragmentation are involved in eliciting nuclear changes, but it is often unclear which proteins must be cleaved (and in which order) to make those changes evident from a morphological point of view [Jänicke et al., 1998]. Since in several cases we know the cleaved protein sequence(s), it is now possible to overexpress mutated, uncleavable variants of key nuclear proteins in order to ascertain the relative contribution given by each proteolyzed polypeptide to morphological changes. So far, however, this approach has only been used for nuclear lamins [Rao et al., 1996]. Similar experiments

may prove difficult to perform if the mutated proteins loose their function(s) and this in turns negatively affects cell proliferation. However, the use of inducible overexpression systems may circumvent such a problem. Given that also post-translational modifications of proteins are likely to play a fundamental role in apoptotic nuclear changes (perhaps by facilitating proteolysis and/or DNA cleavage), overexpression of proteins mutated in amino acid residues critical for the modifications to occur (for example abrogation of sequences targeted by protein kinases) should improve our understanding of the exact role played by these modifications. Therefore, other fundamental steps will be represented by the identification of the protein kinases and phosphatases that are active within the apoptotic nucleus and of how they are regulated, also considering that in the future these enzymes might be useful targets for specific inhibiting drugs, in an attempt to treat patients suffering from disease in which the apoptotic events occur in an inappropriate way. Furthermore, the exact role played by each caspase (and other proteases as well) in the nuclear changes must be defined with accuracy [Slee et al., 2001].

Additional knowledge of nuclear apoptotic modifications might also be important for a better discrimination between necrosis and apoptosis, especially in the context of pathological processes in vivo, which has never been an easy task if ultrastructural analysis could not be employed [Columbano, 1995; Bortul et al., 2001]. Finally, advances in our understanding of nuclear events during apoptosis may also have diagnostic relevance, as measurements of the kinetics of degradation or release of specific nuclear matrix proteins is already a valuable addition to other rapid and accurate flow cytometric parameters (annexin staining and light scatter changes) currently employed for routine discrimination of different modes and temporal patterns of cell death [Dynlacht et al., 1996]. In this connection, it should be emphasized that nuclear matrix protein and DNA, both derived from apoptotic cells, are detectable in serum from cancer patients [Miller et al., 1992; Jahr et al., 2001]. Thus, their monitoring may also have a prognostic and therapeutic value, indicating if and to which extent the neoplasm responds to anti-tumor drugs that mainly act by inducing apoptosis in cancer cells [Miller et al., 1993; Hughes and Cohen, 1999].

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