Activation of DNA-degrading enzymes during apoptosis

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Cell death by apoptosis requires a precise plan of destruction of DNA and proteins. In this paper, we review the current knowledge on the different DNA-degrading enzymes which are activated in apoptotic cells. The activation of DNases by upstream proteases is also discussed.

Key words: apoptosis, DNases, L-DNase II, proteases, caspases.

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A balance between cell proliferation and cell death strictly regulates tissue homeostasis. Cell death occurs essentially by two distinct pathways: necrosis and apoptosis. Necrosis, which is caused by non-physiological conditions in the cellular environment, is a passive process characterized by cell swelling, rapid disruption of membrane, random degradation of DNA, organelle damage, dilatation of endoplasmic reticulum and cytoplasm vacuolization. In necrotic cells, membrane integrity is lost, leading to the release of cellular content, with resulting inflammation of surrounding tissues.

The definition of apoptosis was first based on a distinct sequence of morphologic features, described in 1972 by Kerr, Wyllie and Currie. Apoptosis occurs during the embryonic development, in tissue turnover, metamorphosis and atrophy of tissues and organs and during sexual differentiation. In other words, it is involved in many stages of tissue development, providing a way of discarding redundant cellular material (McConkey *et al.* 1996; Jacobson *et al.* 1997; Vaux and Korsmeyer 1999). Dysregulation of apoptosis is implicated in the pathogenesis of many human diseases, including neurodegenerative (Honig and Rosenberg 2000) and autoimmune (Rathmell and Thompson 2002) disorders as well as oncogenesis (Lowe and Lin 2000; Tamm *et al.* 2001; Reed 2001). Apoptosis is an active, energy-dependent process that is characterized by a series of typical morphological events, such as membrane blebbing, condensation of nuclear chromatin into sharply delineated masses that become marginated against the nuclear membranes, cell shrinkage, internucleosomal DNA fragmentation and protein cleavage (Bratton and Cohen 2001; Bonanno *et al.*, 2002). Among the components of the apoptotic machinery, some factors proved to be evolutionarily conserved from nematodes to mammals (Chinnaiyan 1999; Cain *et al.* 2002).

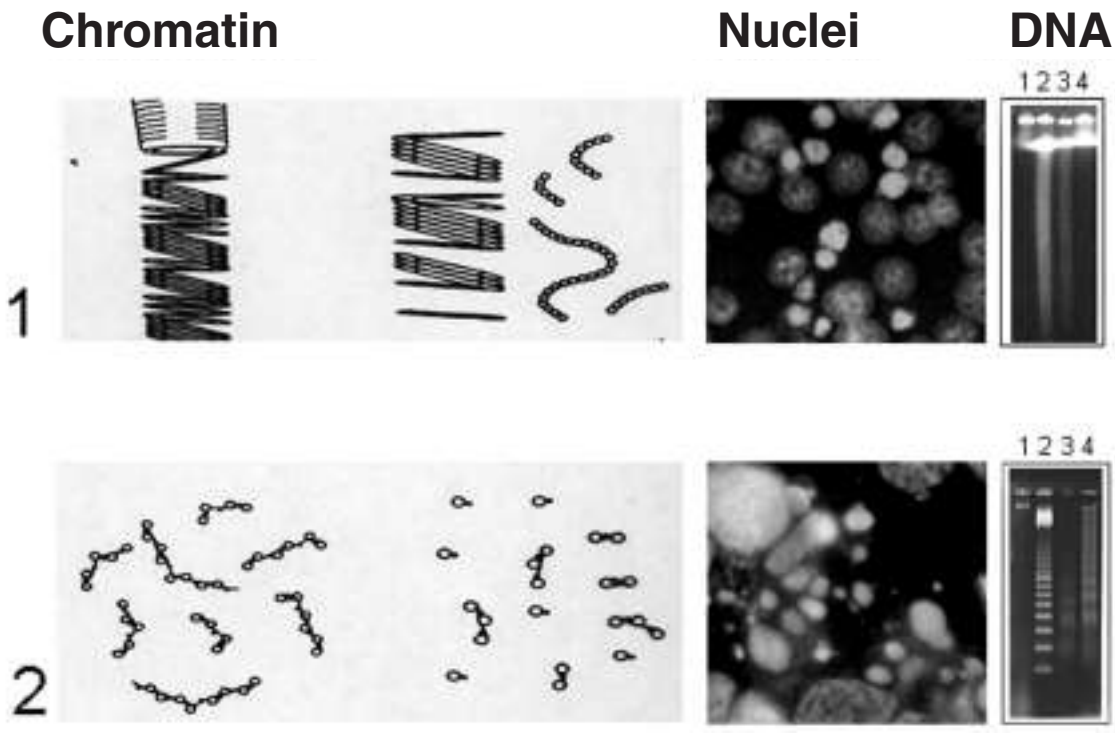

Figure 1. Time-course of DNA degradation in apoptotic cells. Step 1: in nuclei showing chromatin condensation as an incipient sign of apoptosis, DNA is fragmented into high molecular weight fragments (50-300 kb) detectable by pulsed-field gel electrophoresis. Lanes 1,4: control HeLa cells; lanes 2 and 3: HeLa cells treated with 100 μM etoposide for 3 h, and further incubated in fresh medium for 3 h or 24 h, respectively. Step 2: in nuclei showing chromatin fragmentation, internucleosomal DNA degradation occurs, leading to the production of oligonuclesome-sized DNA molecules. A typical DNA ladder is detectable by conventional agarose gel electrophoresis. Lane 1: control HeLa cells; lane 2: markers; lanes 3 and 4: HeLa cells treated with 100 μM etoposide for 3 h, and further incubated in fresh medium for 3 h or 24 h, respectively. Chromatin staining and DNA analysis were performed according to Torriglia et al. (1999).

DNA degradation

Loss of DNA integrity is a common feature of cell death. As an early event during apoptosis, fragmentation of DNA into high molecular weight (HMW) molecules, ranging from 300 to 50 kb, has been described and monitored by pulsed-field gel electrophoresis (Schwartz and Cantor 1984). Following a precise kinetics of DNA degradation, HMW fragments are further degraded to oligonucleosome-sized DNA molecules. The cleavage of DNA at the internucleosomal region generates DNA fragments with lengths corresponding to multiples of 180 bp, giving rise to a typical "ladder" when analyzed by conventional gel electrophoresis (Wyllie 1980; Wyllie *et al.* 1980). A further consequence of DNA fragmentation is the presence of free DNA termini, which can be visualized by the TdT-mediated-dUTP nick end labeling (TUNEL) procedure (Gavrieli *et al.* 1992). The kinetics of DNA degradation is shown in Figure 1.

Internucleosomal DNA fragmentation, a typical hallmark of apoptotic cells (with some exceptions), has been demonstrated to parallel the well-characterized apoptotic morphology in a wide range of situations and cell types (King and Cidlowski 1995). In this respect, the search of DNases, i.e. enzymes cleaving DNA, became a major goal in the characterisation of the apoptotic pathway. This explains the enormous body of literature between 1990 and 1997 concerning endonucleases activation in apoptosis (for reviews, see Montague and Cidlowski 1996; Walker and Sikorska 1997; Counis and Torriglia 2000; Zhang and Xu 2002).

In this paper we will review the current knowledge on the DNases that are activated during apoptosis. A comprehensive review of the literature prompted us to classify these enzymes in three groups, according to their activity dependence (Counis and Torriglia 2000): i) Ca^{2+}/Mg^{2+}-dependent endonucleases; ii) Mg^{2+}-dependent DNases; iii)

acid endonucleases or cation-independent DNases. This classification was chosen because many of these enzymes have not been fully characterized and a structural or genetic analysis have not been achieved.

Ca^{2+}/Mg^{2+}-dependent endonucleases

Among the different Ca^{2+}/Mg^{2+}-dependent DNases, DNase I is the best characterized (Peitsch *et al.* 1993). It has been shown that isolated nuclei of COS cells having little endogenous endonuclease activity, acquire the capability to degrade their DNA into multiples of 180 bp fragments upon transfection of the cells with DNase I cDNA (Polzar *et al.* 1993). Another DNase implicated in apoptosis is DNase g, which has been characterized at the molecular level (Shiokawa and Tanuma 1998a; 2001). The activity of other nucleases was also investigated; for instance, a 18 kDa nuclease, homologous to cyclophilin, has been isolated from rat thymus (Gaido and Cidlowski 1991; Montague *et al.* 1994; Montague and Cidlowski 1996). Wyllie and coworkers have isolated from thymocytes a protein of 110-130 kDa, which is closely related to one subunit of topoisomerase II (Arends *et al.* 1993). Finally, a caspase 3-activated DNase belongs to this category, i.e. CPAN, the human homologue of CAD (Halenbeck *et al.* 1998). The human Ca^{2+}/Mg^{2+}-dependent DNAS1L3, a nuclear enzyme which is able to cleave DNA into large molecular weight and oligonucleosomal fragments (Boulares *et al.* 1999), proved to be regulated *in vitro* and *in vivo* by poly(ADP-ribosylation) (Yakovlev *et al.* 1999; 2000). In this way, the release of DNAS1L3 from poly(ADP-ribosylation)-induced inhibition allows it to mediate DNA fragmentation and cell death. Boulares *et al.* (2001; 2002a,b) demonstrated that cells lacking DFF45, an apoptotic DNA-fragmentation factor, as well as PARP-deficient cells are less sensitive to apoptosis induced by TNF. Interestingly, human osteosarcoma cells depleted of DNAS1L3 do not activate etoposide-induced apoptosis. This property was recovered after transfection with exogenous DNAS1L3, thus suggesting that loss of expression or inactivation of endonucleases might contribute to reduce cell sensitivity to drug-induced apoptosis.

Mg^{2+}-dependent DNases

Mg^{2+}-dependent, Ca^{2+}-independent DNases were characterized in $CD34^+$ cells (Kawabata *et al.* 1993; Anzai *et al.* 1995). The best-studied enzyme of this class is CAD, which is Ca/Mg-dependent in human cells but Mg-dependent in mouse. CAD is the first example of a caspase-activated DNase. Enari *et al.* (1998) purified CAD from mouse lymphoid cells as a protein of 40 kDa. During the purification process, it was noted that the extracts from non-apoptotic cells contained a factor that inhibits the CAD. The factor was purified and designated as ICAD (inhibitor of CAD), which is a substrate of caspase 3 (Enari *et al.* 1998), thus indicating that the activation of endonucleases is downstream caspases. The same factors were purified independently by Liu (called DFF) and Halenbeck (called CPAN) from HeLa and Jurkat cells, respectively (Halenbeck *et al.* 1998; Liu *et al.* 1998). DFF is composed of two subunits of 45- and 40-kDa (DFF-45 and DFF-40). Caspase 3 cleaves DFF-45 and generates a DNase activity, so that DFF-45 is the equivalent of ICAD and DFF-40 is the equivalent of CAD. As well as inhibiting CAD, ICAD seems to have chaperone properties in regard of CAD, since in its absence no active CAD can be produced (Sakahira *et al.* 2000). CAD and ICAD are expressed in most tissues and cell lines undergoing rapid DNA fragmentation after apoptotic stimuli (Mukae *et al.* 1998). In contrast, some cell types like fibroblasts and nerve cell lines have low levels of these proteins. Zhang *et al.* (1999) showed in thymocytes from ICAD-null mice no DNA fragments upon exposure to apoptotic stimuli, thus demonstrating that CAD/ICAD system regulates DNA fragmentation.

Acid endonucleases or cation-independent DNases

Eastman's group was the first to implicate acid DNases in DNA fragmentation during apoptosis (Eastman and Barry 1992; Barry and Eastman 1993; Barry *et al.* 1993). Other authors showed that intracellular acidification induces DNA cleavage that may be catalyzed by these types of DNases (Gottlieb *et al.* 1995; 1996; Collins *et al.* 1996; Leaseholder *et al.* 1996; Furlong *et al.* 1997). Different groups have cloned a DNase II (Baker *et al.* 1998; Krieser and Eastman 1998; 2000; Shiokawa and Tanuma 1998b; Wang *et al.* 1998; Yasuda *et al.* 1998; Lyon *et al.* 2000) that seems to be a secreted enzyme (Wang *et al.* 1998). Recent work using knock-out mice for DNase II indicates that this enzyme is important for housekeeping during erythroid cell differentiation and DNA clear-

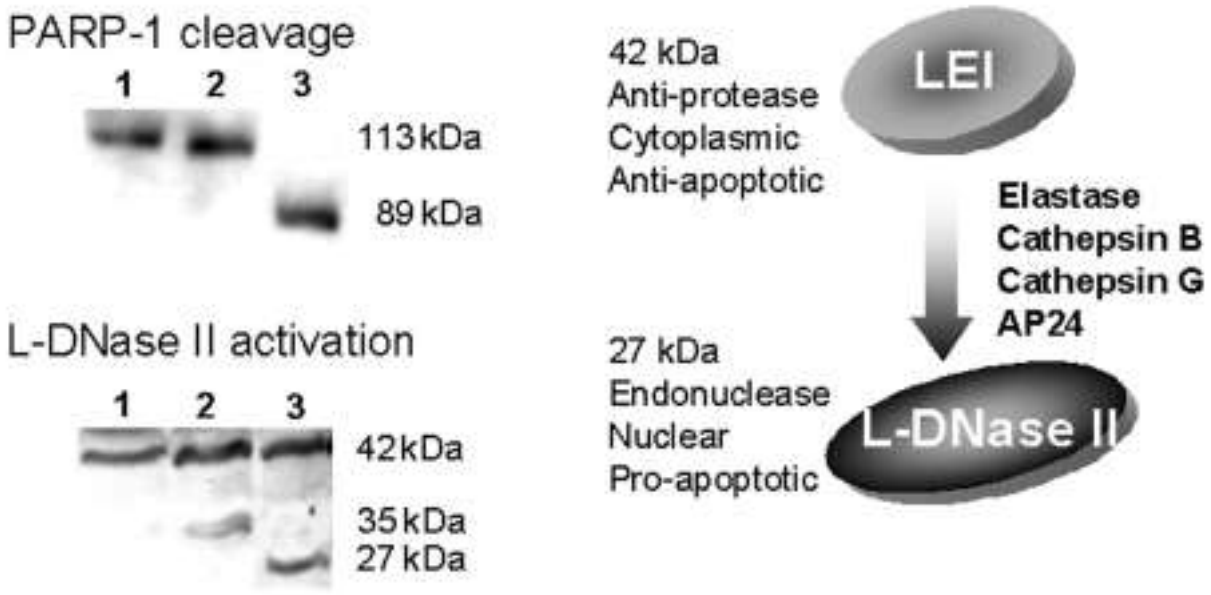

Figure 2. Conversion of LEI (Leukocyte Elastase Inhibitor) into L-DNase II in apoptotic cells. Total extracts from long term-cultured HeLa cells (i.e. cells grown in the same medium for 8 days) were analyzed by western blot for PARP-1 cleavage and for L-DNaseII activation. Lane 1: control cells; lane 2: attached cells recovered after 8 days in culture; lane 3: floating, apoptotic cells recovered after 8 days in culture. Apoptotic cells (lane 3) are characterized by the presence of PARP-1 cleavage product of 89 kDa, and of the 27 kDa L-DNase II. Western blot analysis was performed according to Torriglia *et al.* (1999). Right panel shows the features of LEI and L-DNase II, and the possible mechanisms regulating the conversion of LEI into L-DNase II.

ance, but not for cellular apoptosis itself (Kawane *et al.* 2001; Krieser *et al.* 2002). In 1999, an acid nuclease, activated during apoptosis in HeLa cells, was found (Famulski *et al.* 1999). This nuclease is active in acetate buffer and insensitive to Zn^{2+}, and its activation is caspase-independent. In our laboratory, we have demonstrated the involvement of DNase II in chick lens cell terminal differentiation (Torriglia *et al.* 1995), which is characterized by the disappearance of nuclei from lens fiber cells. The process of nuclear degradation shares many features with nuclei of apoptotic cells (Counis *et al.* 1998), even if DNA fragments are not a good substrate for TUNEL reaction (Chaudun *et al.* 1994). Since DNase II cuts DNA and produces 3'P termini, we evaluated the involvement of this enzyme in the apoptotic pathway and we showed that antibodies directed against DNase II inhibit DNA degradation in isolated lens cell nuclei. Remarkably, DNase II is located in the cytoplasm of undifferentiated cells and only becomes nuclear in cells undergoing differentiation.

L-DNase II

Recently, we have characterized a protein with a DNase II activity, which is different from the above described DNase II (Torriglia *et al.* 1998). *In vitro* this DNase, called L-DNase II, has the properties of a DNase II, with optimal activity at pH 5.75. In contrast to the other DNase II described above, it keeps the 50% of its activity at pH 7.4 (Counis *et al.* 1998). L-DNase II is derived from LEI (Leukocyte Elastase Inhibitor) by an acid-dependent post-translational modification or by digestion with elastase. *In vitro* experiments, using purified recombinant LEI, showed that the native form has no activity on purified nuclei whereas its post-translationally activated form induces picnosis and DNA degradation in isolated nuclei. The conversion of LEI into L-DNase II is promoted by intracellular acidification (Altairac *et al.* 2003a). In different cell lines, we showed an increased expression and a nuclear translocation of L-DNase II during apoptosis (Torriglia *et al.* 1999; Belmokhtar *et al.* 2000; Gorrini *et al.* 2003). In Figure 2, western blot analysis of the conversion of LEI into L-DNase in apoptotic cells is reported. A typical example of intracellular redistribution of L-DNase II during apoptosis is shown in Figure 3.

The activation of DNases during apoptosis

The apoptotic sequence proceeds according to a precise plan of destruction of DNA and proteins. As above reviewed, DNA can be degraded by many

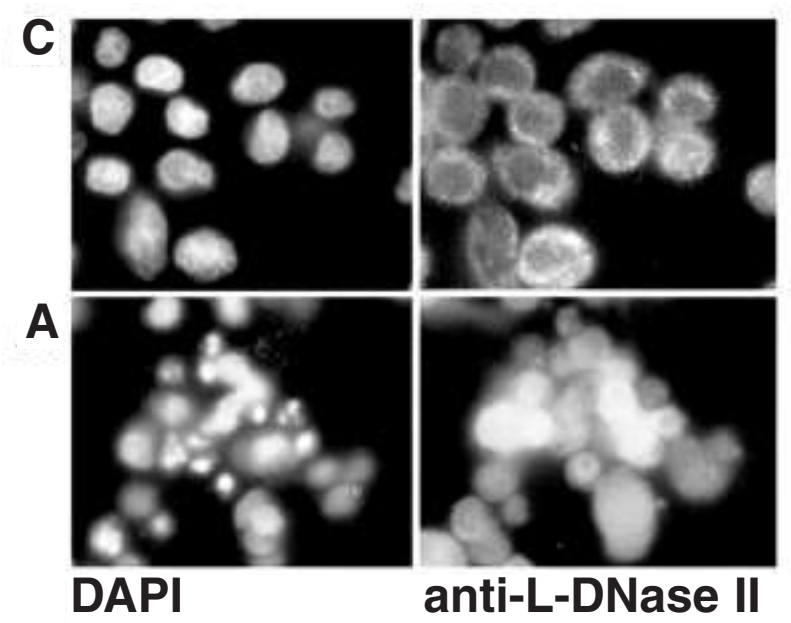

Figure 3. Translocation of L-DNase II from the cytoplasm to the nucleus of apoptotic HeLa cells. In control cells (C), L-DNase II shows an extra-nuclear localization. In long term-cultured cells, L-DNase II immunofluorescence is visible in the nuclear compartment. Immunofluorescence was performed according to Torriglia *et al.* (1999).

DNases/endonucleases, possibly following different strategies, depending on the cell type and the nature of the stimulus. The activity of DNA-degrading enzymes is in turn regulated by proteases. Consequently, CAD is activated after cleavage of ICAD, its inhibitor, by caspase 3 (Enari *et al.* 1998). If we assume the other endonucleases activated during apoptosis behave in the same way, a class of non caspase-proteases should exist and activate, directly or not, other endonucleases. This seems to be the case, since other proteases have been implicated in apoptosis.

The proteases activated during apoptosis

Caspases

PARP-1, the best marker of proteolysis during apoptosis (Scovassi and Poirier, 1999; Soldani and Scovassi, 2002), proved to be cleaved in cells treated with different drugs (Kaufmann *et al.* 1993; Soldani et al., 2001), by a *protease resembling ICE* (prICE; Lazebnik *et al.* 1994). This protease was further identified as caspase 3 (Nicholson *et al.* 1995; Tewari *et al.* 1995; Casciola-Rosen *et al.* 1996). At least 14 mammalian caspases have been described (Chang and Yang 2000). Caspases exist in the cell as pro-caspases, whose activation requires a proteolytic event and occurs in a cascade-like way. In respect to the time-course of their activity during the apoptotic pathway, they can act as initiator or effector caspases. Once activated, the initiator caspases process downstream caspases to promote the cleavage of a number of substrates (Salvesen and Dixit 1997; Thornberry and Lazebnik 1998; Chang and Yang 2000; Cain *et al.* 2002). The use of inhibitors and gene knock-out strategy revealed that caspases have overlapping roles and that their function is so essential that they are redundant within the cell. Many targets of caspases have been described, including structural proteins, enzymes and cell cycle factors. That caspase inhibition does not abolish cell demise, suggests the existence of a caspase-independent apoptotic pathway (Leist and Jäättelä 2001). In this respect, a number of alternative proteases have been described to be active during apoptosis, as reported below.

Calpains

Calpains are non-lysosomal Ca^{2+}-dependent cysteine proteases structurally unrelated to caspases. Ubiquitous cytoplasmic calpains exist as two isoforms, μ-calpain and m-calpain, and are constitutively expressed. Their possible involvement in apoptosis was first suggested by Sarin *et al.* (1993) and Robert-Lewis *et al.* (1993) and has been reported for several cells, even if little is known about their exact role. By the use of calpain inhibitor acetyl-calpastatin 27-peptide, it has been clearly demonstrated that calpains are required after a number of apoptogenic stimuli (Sarin *et al.* 1994; Squier *et al.* 1994; 1999; Squier and Cohen 1997), including during calcium-dependent apoptosis (Ravid *et al.*

1994; Gil Parrado *et al.* 2002). Interestingly, the overexpression of calpastatin gene reduces dystrophic pathology, thus suggesting that calpain inhibition might provide a therapeutic strategy for this disorder (Spencer and Mellgren 2002), as well as for Alzheimer (Di Rosa *et al.* 2002) and Huntington (Goffredo *et al.* 2002) diseases. Calpain substrates are cytoskeleton proteins including fodrin, membrane receptors and transporters, and steroid receptors (Chan and Mattson 1999). It is noteworthy that Bcl-xL is converted by calpain from the anti-apoptotic into the pro-apoptotic form (Nakagawa and Yuan 2000) and that Bid cleavage promotes cytochrome c release (Chen *et al.* 2001). By proteolytic degradation, calpains also contribute to the regulation of P53 (Pariat *et al.* 1997) and c-Myc (Small *et al.* 2002) levels during apoptosis. Remarkably, a crosstalk between calpains and caspases during apoptosis was postulated. In fact, calpain is implicated in caspase-7 activation during B cell clonal deletion (Ruiz-Vela *et al.* 1999) and is responsible for cleaving pro-caspase 12 to generate active caspase-12 (Nakagawa and Yuan 2000; Neumar *et al.* 2003). To date no relationship has been found between the activation of calpains and a particular endonuclease.

Cathepsins

The cathepsin protease family includes cysteine, aspartate and serine proteases (Chapman *et al.* 1997). Cysteine cathepsins L and B, together with the aspartic protease cathepsin D, are the most abundant lysosomal proteases and have been clearly correlated with apoptosis (Roberts *et al.* 1999; Tsukuba *et al.* 2000; Uchiyama 2001). Genetic evidence for the role of cysteine cathepsins in apoptosis has been provided by studies showing resistance against TNF-induced apoptosis in mice lacking cathepsin B (Roberts *et al.* 1999; Guicciardi *et al.* 2000; Felbor *et al.* 2002), and massive death in the brains of mice that lack cathepsin inhibitor cystatin B (Lieuallen *et al.* 2001) and in cells treated with cathepsin inhibitors (Castino *et al.* 2002). Cathepsins are translocated from lysosomes to the cytosol and/or to the nucleus before the appearance of apoptotic morphological changes. Some of them are involved in caspase-dependent apoptosis (Turk *et al.* 2000; Roberg *et al.* 2002) and cleave crucial factors, such as Bid (Stoka *et al.* 2001) and Brm (Biggs *et al.* 2001).

Granzymes

Granzymes, a family of serine proteases, are packaged in the granules of CTLs and natural killer cells (Trambas and Griffiths 2003). Granzyme B, the most powerful pro-apoptotic member of the family, after intracellular delivery by perforin induces a proteolytic cascade by acting as an apical caspase, thus processing a number of key caspases (Atkinson *et al.* 1998; Barry *et al.* 2000). Granzyme B is also able to cleave and activate directly the pro-apoptotic factor Bid (Pinkoski *et al.* 2001; Sutton *et al.* 2000) and other proteins in a caspase-independent death pathway. In fact, in the presence of caspase inhibitors, granzyme B might cause cell death independently of the caspases, even with a slower kinetics. Death induced by granzyme A is associated with DNA single-strand breaks created by a granzyme A-activated DNase (Beresford *et al.* 2001; Fan *et al.* 2003).

Serine proteases

Other serine proteases different from granzymes have been described to play a role in cell death: Omi/Htra2, a mitochondrial protease that upon induction of apoptosis is released to the cytoplasm, is involved in both caspase-dependent and -independent apoptosis (Hegde *et al.* 2002; Cilenti *et al.* 2003; Jin *et al.* 2003). The 24 kDa apoptotic protease (AP24) is a serine protease with elastase-like activity that is activated during TNFα- or UV light-induced apoptosis and stimulates *in vitro* internucleosomal DNA fragmentation in isolated nuclei (Wright *et al.* 1997; 1998a; 1998b). We recently described the interaction between AP24 and LEI/L-DNase II (Altairac *et al.* 2003b) and showed that L-DNase II is activated in U937 cells treated with TNFα, i.e. in an apoptotic model recruiting AP24. Remarkably, we found that L-DNase II activity is suppressed when apoptosis is attenuated by an AP24 inhibitor, carbobenzoxy-Ala-Ala-borophe (DK120).

DNases and the control of apoptosis

DNA degradation during apoptosis has often been considered a housekeeping task in the cell. However, recent findings show that DNA degradation can also control apoptosis. Parrish *et al.* (2001), while searching for new cell death-inducing factors that are released from cells primed to die, purified endoG, a previously described mitochondrial enzyme with a proposed role in the replication of

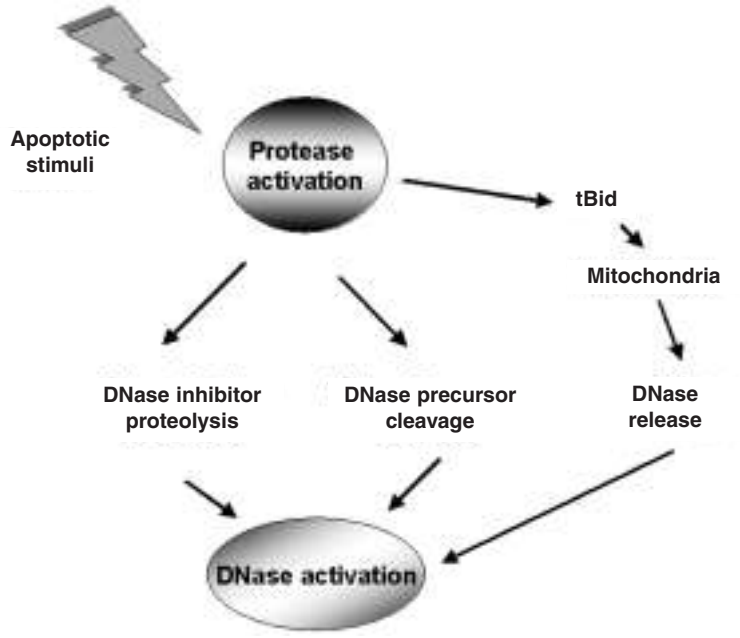

Figure 4. Cross-talk between proteases and DNases during apoptosis. Active proteases, including caspases, calpains, cathepsins, serine proteases, can promote the activation of DNases in different ways, i.e. by cleaving a DNase precursor (LEI/IL-DNase II) or a DNase inhibitor (CAD/ICAD; PARP-1/NAS1L3) or by releasing DNases from mitochondria (endoG).

mitochondrial DNA. These authors suggested that endoG not only participates in the *deconstruction* of apoptotic cells, but can also contribute to the actual killing process. These authors identified the genes that, when mutated, protect cells from apoptosis. Among them, cps-6, (the nematode counterpart of endoG) not only promotes DNA degradation, but also contributes to cell killing. Its loss slightly increased cell survival in many genetic backgrounds of *C. elegans*. These results suggest that DNA degradation and therefore endonucleases have a role in the control of apoptosis, perhaps because of the importance of their housekeeping function.

Conclusions

Over the two decades following the pioneering work of Kerr *et al.* (1972), it was generally accepted that different apoptotic stimuli activate a common apoptotic pathway. This idea was supported essentially by the fact that the apoptotic process is highly conserved from the morphological point of view, and because of the discovery of caspases as common executioners of apoptosis. More recently, the discovery of many DNases involved in DNA degradation in different apoptotic systems, and of proteases other than caspases, have shown that distinct molecular pathways lead to apoptosis. In this respect, it is now widely accepted that caspase-dependent and -independent pathways exist, leading to alternative execution systems. Factors involved in caspase-independent apoptosis are under characterization (Nicotera 2001; Mathiasen and Jäättelä 2002; Jäättelä and Tschopp 2003). To date, a precise link between endonucleases and the non caspase-proteases has not been stated, except for LEI/L-DNase II, that may be activated by AP24, a serine protease.

From these facts, an intriguing question arises: why would a cell need more than one nuclease to digest its DNA? One hint might come from the different ways of regulation of such enzymes (Figure 4). In living cells, CAD is sequestered in an inactive complex, in a form bound to its inhibitory subunit (known as ICAD or DFF45). By contrast, endoG is activated by a change in its subcellular localization and L-DNase II derives from a protein which has another function (LEI is a protease inhibitor). The existence of redundant pathways leading to cell death may help when caspase activation is limited or compromised, as it might be the case, for example, during viral infection.

Acknowledgements

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