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Molecular mechanism of L-DNase II activation and function as a molecular switch in apoptosis

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ABSTRACT

The discovery of caspase activation counts as one of the most important finds in the biochemistry of apoptosis. However, targeted disruption of caspases does not impair every type of apoptosis. Other proteases can replace caspases and several so called ''caspase independent'' pathways are now described. Here we review our current knowledge on one of these pathways, the LEI/L-DNase II. It is a serine protease-dependent pathway and its key event is the transformation of LEI (leukocyte elastase inhibitor, a serine protease inhibitor) into L-DNase II (an endonuclease). The molecular events leading to this change of enzymatic function as well as the cross-talk and interactions of this molecule with other apoptotic pathway, including caspases, are discussed.

1. Apoptosis and caspase activation

In 2005 the Nomenclature Committee on Cell Death (NCCD) decided that the 'official' classification of cell death had to rely on morphological criteria owing to the absence of a clear-cut equivalence between structural alterations and biochemical pathways [1]. Cell death classified as apoptosis is classically performed by a genetic-determined pathway first identified in the nematode *Caenorhabditis elegans* in H. Robert Horvitz' laboratory [2–4]. Further studies identified caspases as mammalian counterparts of these genes [5,6]. Studies of nerve growth factor deprivation suggested that the 'point-of-no-return' (step in the signalling process after which termination of the inducing stimulus does not prevent the execution of apoptosis) coincides with caspase activation [7,8]. Afterwards caspase activation has been seen as the biochemical marker of apoptosis, a point of view that is still supported by some authors [9]. Although this is sometimes applicable, we think, in accord with others [1,10,11], that this is an inexact generalisation. Apoptosis can be achieved without caspase activation, and caspase activation does not necessarily cause cell death [12]. So that, caspase-3 knockout mice [13–15] have excess of brain tissue, but cell death in other organs is less affected.

2. The caspase-independent apoptosis: proteases and DNases

Caspases are activated through the so-called extrinsic and intrinsic pathways (for a review see [9]). The intrinsic pathway is activated through cytochrome c release from the mitochondria

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and built up of the apoptosome, a macromolecular complex formed by cytochrome c, procaspase-9 and the apoptotic factor 1 (Apaf-1). In embryonic fibroblasts from Apaf-1 KO mouse, overexpression of BH3-domain only proteins (pro-apoptotic members of the Bcl-2 family) allows the emergence of an apoptotic morphology but no caspase activation was detected [16]. As caspases are proteases, it is presumable that caspase-independent cell death might require other proteases. Indeed, suggestive evidence supports roles for cathepsins, calpains and other proteases in caspase-independent cell deaths.

Calpains are calcium sensitive cysteine proteases. Two forms of calpains, μ-calpain and m-calpain (also called calpains I and II, respectively), are ubiquitously expressed in human cells. They can be activated by calcium influx. Recently, these enzymes have been implicated in both pro- and anti-apoptotic functions. *Capn* 4−/− (gene coding for the regulatory subunit of each calpain) MEFs cells were resistant to puromycin, camptothecin, etoposide, hydrogen peroxide, ultraviolet light, and serum starvation, but were more sensitive to staurosporine and tumor necrosis factor [17]. Calpain activation has been associated with several neurodegenerative diseases [18], like Alzheimer's disease and Huntington's disease [19]. During trophic factor deprivation in sympathetic neurons, calpains cleave Bax into a pro-apoptotic 18-kDa fragment which promotes cytochrome c release and apoptosis [20].

Cathepsins form a group of proteases found predominantly in lysosomes [21]. Interdigital cell death occurring during limb development provides an interesting example of massive cell death in development [22]. In this paradigm, cell death is not inhibited in mice deficient in either caspase-2, -3, -6, -7, -8 or -9 [23–26], despite their activation in these cells [22]. The combined administration of pepstatin A and Z-VAD.FMK inhibits cell death more strongly than the inhibitors used separately, thus suggesting that both caspases and cathepsin D participate in this process [22]. Although cathepsins activities are partly regulated by the unfavourable neutral pH in cytosol, their endogenous inhibitors, stefins, cystatins, serpins and thyropins, constitute the major defense mechanism [27].

Several serine proteases have been individually implicated in cell death: granzymes [28,29], Omi [30], AP24 [31]. Stenson-Cox et al. showed recently that the pan-caspase inhibitor Z-VAD.FMK does not prevent all apoptotic features in staurosporine-treated HL-60 cells, while it abrogated caspase-3 activation and cleavage of PARP-1 [32]. Only chymotrypsin-like inhibitors and a pan-serine protease inhibitor could significantly reduce cell shrinkage, nuclear condensation and oligonucleosomal DNA degradation. However, it did not prevent PARP-1 cleavage, suggesting that serine proteases are activated in parallel but independently of caspases. Moreover, this group has also recently characterised three putative serine proteases involved cell death programmes [32].

During cell death, the central component of the controlled destruction of the cell is the proteolytic system that disassembles the cell. This dismantling is completed by the action of endonucleases that will destroy the potentially harmful DNA. Actually, nuclease activity is important for the commitment to apoptosis and the inhibition of DNA cleavage delays apoptosis execution [11,33–35].

In the early 90s the characterisation of a DNase involved in apoptosis became a major goal in the description of this pathway [36,37]. Several enzymes were proposed as candidates, including DNase I, DNase II, cyclophilins, and DNAS1L3 [38].

None of them, however, appeared to fulfil the criteria for the apoptotic DNase, i.e. being activated by proteases in the onset of apoptosis. In 1998 Nagata's group identified CAD (caspase-activated DNase), an endonuclease that degrades DNA and that is activated by caspases [34,39]. CAD is synthesised with a chaperone, ICAD (inhibitor of CAD), which is also its inhibitor. After activation of caspase-3, ICAD is cleaved, releasing CAD that will degrade DNA.

If we assume that the other proteases activated during apoptosis may also promote DNA degradation, the proteases we mentioned above should activate other endonucleases. However, the links between these different enzymes is not fully elucidated. Very few endonucleases were linked to the activation of proteases. Actually two endonucleases have been related to non-caspase proteases activation. These are GAAD (granzyme A-activated DNase), discovered by the Lieberman's group [40–42], and L-DNase II (LEI-derived DNase II).

3. LEI/L-DNase II discovery in the lens

In the early 90s three different endonucleases were involved in DNA fragmentation leading to nucleosomal production. Some authors, as Peitsch et al. [43], claimed that the well-characterised pancreatic deoxyribonuclease (DNase I) was constitutively expressed in cells of tissues potentially primed for apoptosis. But Barry and Eastman [44] were unable to detect a Ca^{2+}–Mg^{2+}-dependent endonuclease while studying apoptosis in Chinese hamster ovary (CHO) cells. Instead, they identified an acid endonuclease, cation-independent, the DNase II. Finally, the group of Cidlowski identified NUC 18, a molecule related to cyclophilin [45]. By this time, CAD had not been identified and it appeared quite obvious from the above works that there was conflicting evidence on the enzymes responsible for the nucleosomal ladder formation.

The lens is a very interesting model for the study of DNases. This avascular organ, localized in the posterior segment of the eye, is composed of a layer of epithelial cells overlaying a concentric array of elongated cells, the fibers. The whole organ is surrounded by the lens capsule. In the equatorial region of the lens, the epithelial cells sequentially differentiate into fiber cells (Fig. 1). During differentiation, the cell elongates, starts the synthesis of crystallines and degrades its organelles-like nucleus and mitochondria [46]. One of the most striking features of lens cell differentiation is the loss of lens cell nuclei presenting the phenomena classically described in apoptotic cell nuclei. The chromatin appears condensed, the DNA is cleaved between nucleosomes, and some histones and HMG proteins are lost, probably contributing to the action of endonucleases on linker DNA. In this tissue, however, the degradation of nuclei is not present in scattered cells, but is highly synchronized [47]. In chicken lens, there was no accumulation of single strand (SS) breaks with free 3′ OH ends and TUNEL technique was negative until a very advanced state of degradation, leading to the hypothesis that an enzyme

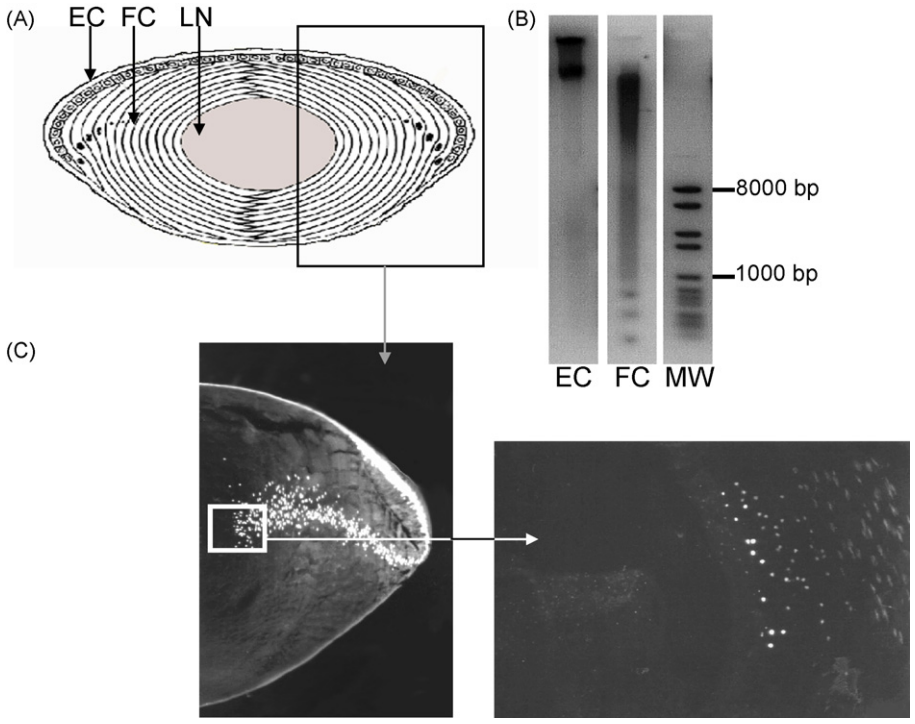

Fig. 1 – DNA degradation during lens cell differentiation. In lens, DNA is degraded in an "apoptotic-like" manner. Panel A shows a schematic representation of a lens illustrating the epithelial, non-differentiated cell (EC), the fiber cells (FC) and the lens nucleus (LN), a zone where the nuclei and the other organelles have disappeared. Panel B is an agarose gel showing the state of DNA in epithelial cells and fibre cells. In fibre cells, DNA degradation and oligonucleosomes production are seen. Panel C shows a DAPI stained section of an 18-day-old embryonic chicken lens (left panel) and a TUNEL stained section (right panel) of the same lens. Only the inner nuclei are stained.

generating 3′P ends was responsible for DNA cleavage [48]. In order to investigate this point we generated antibodies against DNase II and showed that this enzyme localizes to the cytoplasm of epithelial, non-differentiated cells, but translocates to the nucleus in fiber cells [49]. Moreover, antibodies against DNase II were able to block DNA degradation in fiber cells. All this features raised our interest for this enzyme, further work allowed us to isolate, sequence and characterise the protein responsible for this effect. In 1998 we characterised an enzyme that we called L-DNase II because it was different from DNase II, which had been isolated by the Eastman's group the same year [44], and because we showed that it is derived by post-translational modification from leukocyte elastase inhibitor (LEI). LEI, also known as serpin B1 belongs to the serpin (*ser*ine *p*rotease *in*hibitors) superfamily and it can be classified among the ovalbumin serpins or clade (subfamily) B serpins [50–52]. The members of this clade are, contrary to all other clades, predominantly intracellular. Like most serpins, LEI has an anti-protease activity [53]. In its native form, it inhibits elastase, cathepsin G and proteinase 3 [54]. LEI may be post-translationally modified into L-DNase II, either by exposure to an acidic pH or by the action of proteases like elastase. Treatment of LEI by these agents is linked to a decrease of its apparent molecular weight, to a loss of its anti-protease activity and to the appearance of an acid endonuclease activity [53]. This apparent contradiction is explained by the mechanism of protease inhibition as explained in the following sections.

Many studies performed in different cellular and in vivo models, confirmed the activation of this system during cell death as will be discussed in the following paragraphs.

4. LEI/L-DNase II in apoptosis

By the time of L-DNase II discovery, we were investigating the activation of DNases in different apoptotic paradigms. We found that when HeLa cells were induced to undergo apoptosis by long-term culture, both Ca–Mg-dependent DNases and acid DNases were activated [55]. Caspases inhibitors completely inhibited Ca–Mg-dependent endonucleases but had no effect on acid endonucleases, and no effect on apoptosis. It seemed then that induction of apoptosis in HeLa cells by a metabolic stress is triggered by a caspase-independent pathway and that activation of acid DNases during apoptosis is also independent of caspases. The activation of acid DNases has also been seen in other physiological models such as neural apoptosis during retina development [56] or in cell culture [32,55,57–61] as well as in pathological conditions like corneal graft rejection [62,63], and light-induced retinal degeneration [64]. In all the cases, the activation of this pathway seems to be triggered by a metabolic

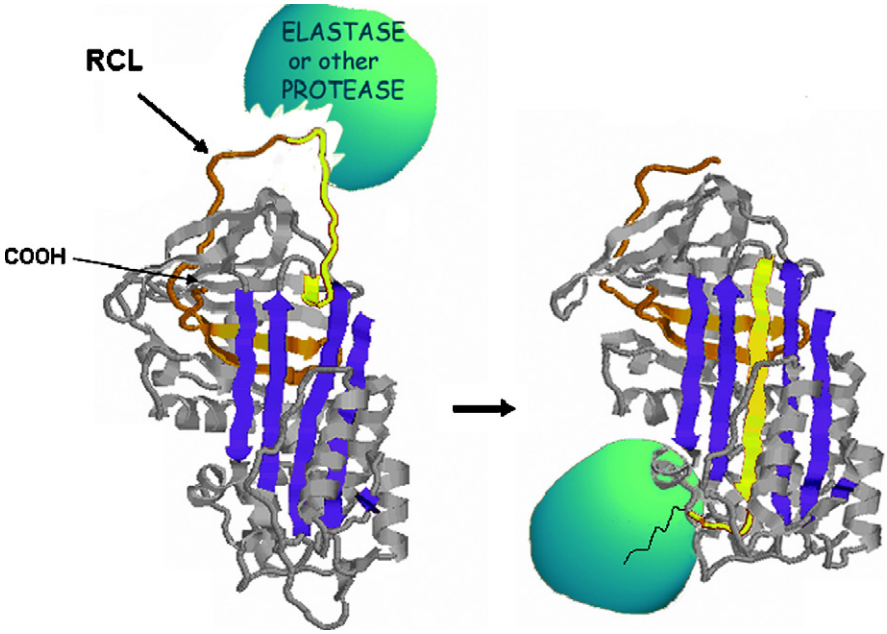

Fig. 2 – LEI structure. LEI structure is represented in a ribbon configuration using Protein Explorer. Native LEI (left panel) exhibits the reactive center loop (yellow and orange strand arrowed RCL). This three-dimensional structure was obtained from pig LEI sequence. Right panel shows cleaved horse LEI (PDB number 1HLE) after cleavage by elastase. Note the insertion of the RCL in the Aβ-sheet of LEI. This conformational change translocates the protease to the other pole of the anti-protease, trapping it in a covalent link.

stress; indeed, we have never seen the activation of this pathway by a genotoxic stress unless a metabolic stress is also arising [61]. It is mostly seen in differentiated cells or in tumor cells having an impairment of the caspase pathway [58,60,65–68]. In all these models dying cells showed morphological characteristics of apoptosis, e.g. cellular and cytoplasm condensation, chromatin condensation, blebbing and apoptotic bodies production.

5. The molecular mechanism transforming LEI into L-DNase II

As evoked previously, the post-translational modification transforming LEI into L-DNase II reduces the apparent molecular weight of LEI. As any serpin, LEI is a protease inhibitor that acts by a suicide mechanism; it interacts with its target protease in a substrate-like manner and inhibits it by a tight-binding mechanism. Its ability as a protease inhibitor depends on a physicochemical property: its metastability, i.e. its native conformation is not the most stable from a thermodynamic point of view. So that, when the reactive center loop (RCL) of LEI is cleaved and an acyl-enzyme complex is produced with the protease, a conformational change leads to the insertion of the RCL into the center of the existing β-sheet A [69]. This conformational change traps the proteases in a stable covalent complex (Fig. 2) [70–72].

The introduction of a single base mutation in the hinge region of LEI induces a loss of its anti-protease activity [73,74], but the endonuclease activity is not modified [73], indicating that endonuclease and anti-protease activities are physically independent in the molecule. Moreover, this also suggests that the insertion of the RCL is involved in the appearance of the endonuclease activity.

The examination of the distribution of electrical charges at the surface of LEI reveals a quite polarized molecule. This polarization is largely increased by the cleavage of the RCL that uncovers a basic region (Fig. 3). Although there is no consensus sequence for endonuclease activity [75], two aminoacids, aspartic acid and histidine (DH), are very frequently localized at DNase active sites and the histidine moiety seems essential for this activity. It is interesting to note that LEI presents such a doublet and that it is exposed on the surface of the molecule only after cleavage of the RCL. Moreover, it is worth noting that it is surrounded by a ring of basic aminoacids, that may constitute a favourable environment for DNA-binding [75]. Mutation of the histidine moiety of this doublet (His 368, Fig. 4, panel A) completely abolishes endonuclease activity of L-DNase II [73].

The change in the enzymatic activity of LEI induced during its transformation into L-DNase II is also followed by a change in its cellular localization, i.e. L-DNase II is translocated to the nucleus [53] to meet its new substrate, DNA. Thus we analysed the LEI structure looking for a nuclear localization signal (NLS). We found a consensus bipartite NLS in positions 212–213 and 224–226 (Fig. 4A). We systematically mutated these lysines into alanines, leading to the generation of 31 constructs with 1–5 mutated lysines in different combinations. Analysis of the cells expressing these mutants allowed us to classify mutants as having a ''normal'' or an ''altered'' nuclear translocation pattern during apoptosis. Moreover, by scoring the mutants

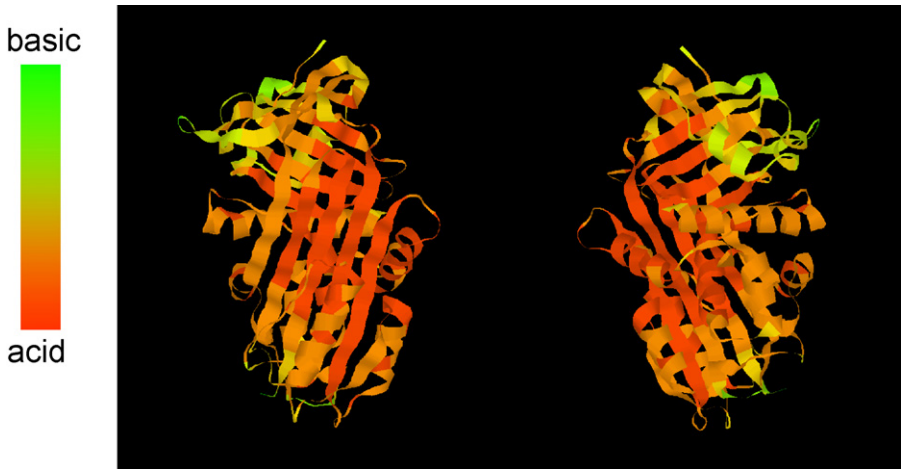

Fig. 3 – LEI is a polarized molecule. Two views of cleaved LEI, coloured according to the aminoacid charge. The upper region of the molecule presents basic charges. It contains the NLS and the endonuclease active site.

impaired for nuclear translocation for a given aminoacid, we evaluated the importance of the different lysines of the NLS (Fig. 4A and B). We have then shown that the most important lysine is the middle one in the group of 3, namely lysine 225. Actually, a single mutation of this central lysine of this group impairs nuclear translocation (Fig. 4B). In addition, using pull down experiments, we were able to show that LEI binds importin alpha and that mutation of lysine 225 into alanine abolishes this interaction [73].

Experiments described above allowed us to conclude that the active endonuclease site of L-DNase II is located underneath the RCL, flanked by a positive charged region that contains a bipartite NLS. Transformation of LEI into L-DNase II is then explained by the conformational modification induced by the cleavage of the RCL, which uncovers the endonuclease active site [73].

One intriguing point of this mechanism is how the cell controls the accession of L-DNase II to the nucleus. As LEI

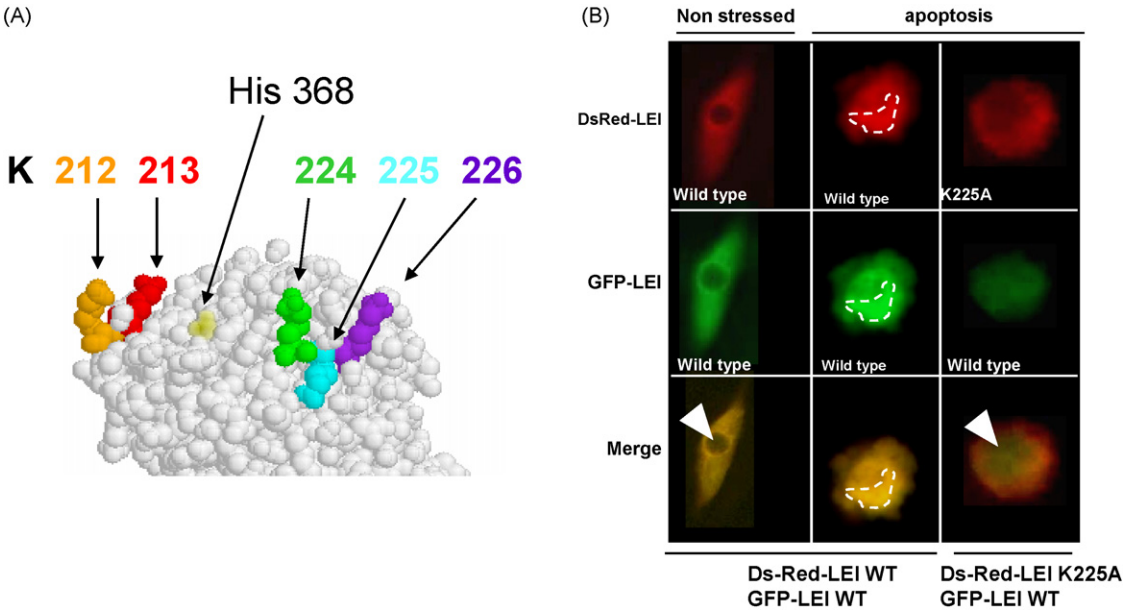

Fig. 4 – The nuclear localization signal. Panel A represents the basic pole of cleaved LEI. The five lysines involved in the NLS (nuclear localization signal) are coloured. Panel B was extracted from Padron-Barthe et al.[73]. It represents a BHK cell transfected with two LEI constructions: in the left column a non-stressed cell transfected with two wild-type constructions, a red fluorescent and a green fluorescent. In the middle panel the same co-transfection is seen in stressed apoptotic cell. Note that in both situations, both constructs perfectly colocalize. In the right column a stressed cell (treated with hexamethylene–amiloride) is seen. This cell overexpress the green fluorescent wild-type construct and a red fluorescent NLS-mutated construction. Note that the red fluorescent construct is excluded from the nucleus. Arrows and dotted line indicate the nuclei.

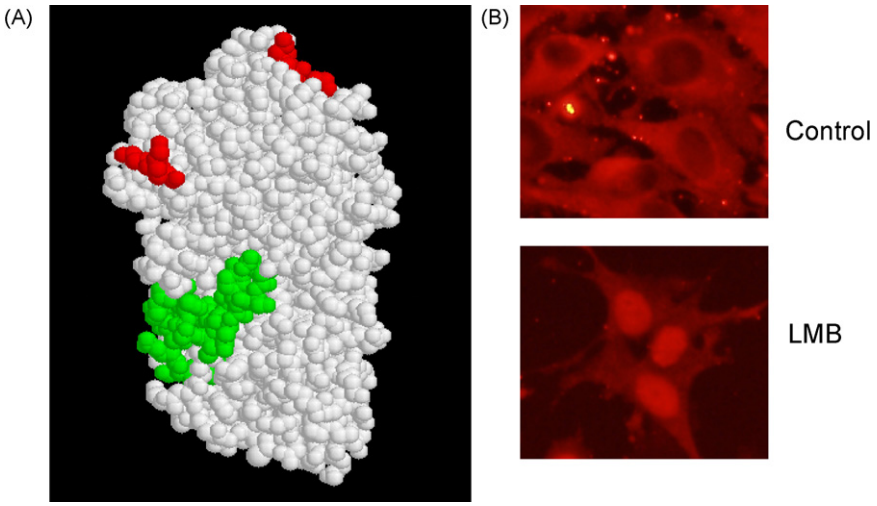

Fig. 5 – Nuclear translocation of LEI/L-DNase II is regulated by a NES. Panel A shows a space-filled representation of cleaved LEI showing the NES in green and the NLS in red. Panel B: the NES binds Crm1 that is responsible for its nuclear extrusion. This receptor is inhibited by Leptomycin B (LMB). The upper panel represents cells stained for LEI in control conditions. In the lower panel the same cells are seen after exposition to LMB.

exists normally in the cell to control protease activity, it is evident that a leakage of L-DNase II must happen in healthy cells. We have recently shown that if L-DNase II does not accumulate in the nucleus, it is because of the presence of a nuclear extrusion signal (NES) [76] (Fig. 5).

The NES consists in a motif containing three or four hydrophobic residues [77]. The NES-dependent nuclear export is mediated by Crm1/exportin. We have found in LEI a consensus NES sequence that binds Crm1, as this receptor is pulled down by an LEI affinity column. In addition, the treatment of the cells with Leptomycin B (LMB), an inhibitor of Crm1, induces LEI accumulation in the nucleus (Fig. 5), confirming that LEI undergoes a nucleo-cytoplasmic shuttling in healthy cells. Site-directed mutagenesis of the aminoacids of this consensus sequence reduces nuclear export of LEI (Fig. 5). The external residues of the NES seem to be more important than the central ones, the most important being valine 295 [76]. This aminoacid is conserved in higher species but is replaced by leucine or methionine – which are also hydrophobic aminoacids – in lower species (chicken and zebrafish). This may be considered as a conservative mutation since CrmA, a viral serpin, presents a leucine in this position and MENT, an heterochromatin-associated serpin, a methionine [78,79].

The nuclear extrusion of LEI/L-DNase II in healthy cells seems to be a very important function. Indeed, the over-expresion of NES-mutated LEI induces cell death. This points out the existence of mechanisms that may be able to regulate the dominance of the NLS on the NES (and *vice versa*) to drive LEI/L-DNase II subcellular localization, depending on the cellular context. As mentioned before, a NES consensus sequence similar to what we have found in LEI, was already described in other serpins of the clade B like CrmA (a serpin from cowpox virus), PI-9 (serpin B9) and maspin (serpin B5) [79]. However, except for maspin, these serpins do not possess a yet identified NLS, and the function of their NES is not clear. As for LEI, these sequences might be functional, but they also might be an evolutionary remnant. Actually, LEI, a protein in which the NES is definitely important, is phylogenetically one of the oldest proteins of clade B serpins [80,81]. Moreover, this sequence could also be important for the folding of these metastable proteins.

6. Proteases transforming LEI into L-DNase II

Upon discovery of L-DNase II, we showed that its activation is favoured at pH 4 but is increased by a tissue extract, indicating that cellular factors might catalyze this transformation. In an attempt to characterise these factors biochemical techniques have been used. S. Altairac, in our group extracted different cells and tissues and found that this activity was highly expressed in spleen extracts. These extracts were then fractioned by using carboxy-methyl cellulose and LEI–sepharose, and two protein fractions were identified. One contained a conversion activity with a neutral optimal pH, the other one contained an activity with an acid optimal pH (Fig. 6). Further studies identified two proteases as being able to transform LEI into L-DNase II: intracellular elastase, a neutral protease, and cathepsin D, an acid protease. Identification of cathepsin D as a target for LEI was quite surprising since this is an aspartate protease (Fig. 6), suggesting that the cleavage site for cathepsin D is different from the one used by elastase (Fig. 6). These acid proteases activities are present in some models of apoptosis activating L-DNase II, as in the embryonic retina [82] and light-induced retinal degeneration (unpublished results) but absent in other models such as in HeLa cells induced to apoptosis by long-term culture. Concerning elastase, the first protease identified *in vitro* as an activator of L-DNase II, we found that this enzyme was activated in two models, i.e. in retina pigment epithelium cells induced to die by ethanol [59] and in murine leukemia cells induced to die by staurosporin [58].

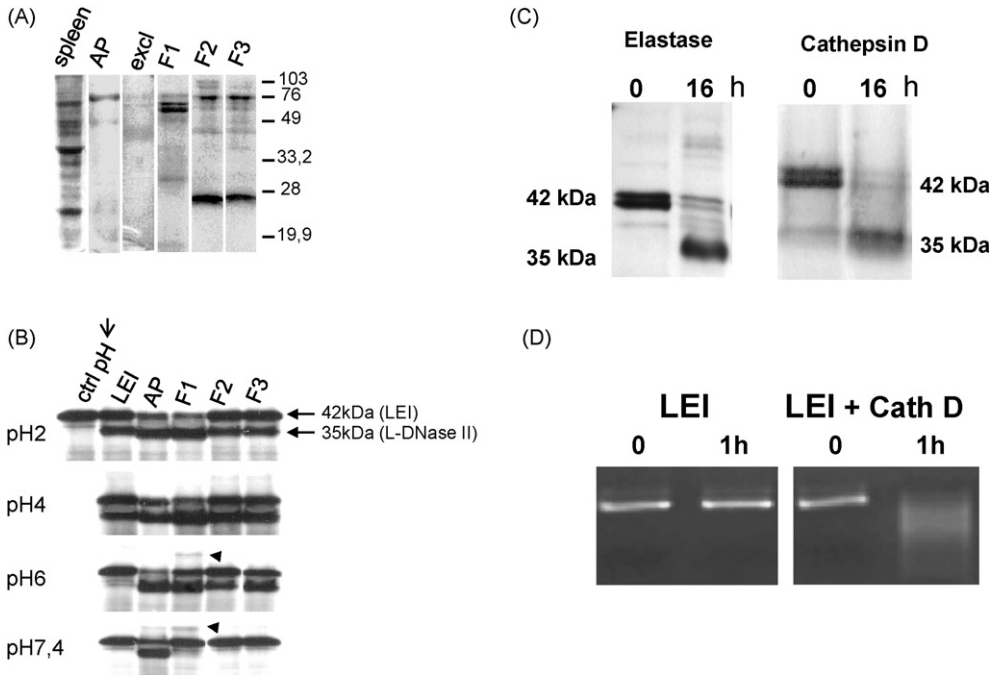

Fig. 6 – Acid proteases can transform LEI into L-DNase II. Panel A: Coomassie blue protein profile of a CM-cellulose column of a pig spleen extract. Total spleen extract (spleen) was acid precipitated by incubation overnight at pH 2.0 (AP). The soluble fraction was loaded on a CM-cellulose column and fractionated into three factions (F1–F3). Panel B: same fractions incubated overnight with a radioactive LEI. Note the presence of the complex in F1 at pH 6 and 7.4 (arrow heads). Panel C: LEI digested with elastase or cathepsin D overnight. Panel D: DNase activity on a supercoiled plasmid of undigested LEI (left) or LEI digested with cathepsin D.

Other elastase-like proteases activated during apoptosis were also shown to be able to activate L-DNase II. This is the case of AP-24 (apoptotic protease of 24 kDa) [83]. This protease is activated during tumor necrosis factor or ultraviolet (UV) light-induced DNA fragmentation in U937 histiocytic lymphoma, BT-20 breast carcinoma, HL-60 myelocytic leukemia, and 3T3 fibroblasts. AP24, is a serine protease, which is able to activate internucleosomal DNA fragmentation in normal nuclei in the absence of cytosol [31]. This feature distinguishes it from several other proteases, such as granzyme B and caspases [34,84], that require the presence of cytosolic components to activate nuclear DNA fragmentation in cell-free systems.

This serine protease is inhibited by carbobenzoxy-Ala-Ala-borophe (DK120) and a DK120 affinity resin was used to purify AP24. We described the interaction between AP24 and LEI/L-DNase II [83] and showed that LEI inhibits AP24 in a serpin-like fashion, forming a stable covalent complex with it and displaying tight-binding kinetics of inhibition. Moreover, after the cleavage of LEI, L-DNase II is activated *in vitro*, and in U937 cells treated with TNFα, an apoptotic model recruiting AP24, L-DNase II is also activated. Remarkably, we found that L-DNase II activity is suppressed when apoptosis is attenuated by a treatment with the AP24 inhibitor DK120, indicating that L-DNase II is the endonuclease activated by AP24.

Other serine proteases may activate L-DNase II. In collaboration with the group of Catherine Stenson-Cox in Ireland, we showed that staurosporie activates a serine protease in parallel with caspase-controlled systems in HL-60 cells. A broad spectrum caspase inhibitor does not affect staurosporin-induced apoptotic morphology, nuclear condensation or DNA fragmentation, despite its prevention of caspase-3 processing and activity. However, inhibitors of serine protease delay these features by some hours. The study of the associated proteolytic activation shows that a chymotrypsin-like protease of 16 kDa is activated and appears to be responsible for specific events downstream of mitochondrial disruption, like generation of L-DNase II and DNA degradation [32].

7. LEI/L-DNase II in molecular models of apoptosis

The activation of L-DNase II was first discovered, as stated before, in the lens during lens cell differentiation [49], which is an apoptosis-related process [47]. In this organ nuclei were TUNEL-negative in spite of the presence of oligonucleosomes in agarose gels. This feature, i.e. the presence of DNA degradation with TUNEL-negative nuclei, is also seen in other cells where L-DNase II is activated during apoptosis, like in endothelial corneal cells [63]. Indeed, the loss of endothelial cells has been observed in human rejected corneal grafts, but the mechanisms of endothelial cell death have remained elusive. In particular it was not clear whether their death was an apoptotic process because TUNEL reaction of these corneal endothelial cells was negative. We showed apoptotic like

nuclear condensation, binding of annexin-V, and apoptotic morphology of the cells that presented important amount of blebs. In this paradigm we have detected the presence of LEI confined to the cytoplasm in endothelial cells when no clinical signs of graft rejection are seen, but it is translocated to the nucleus after 10 days of transplantation, when rejection is observed. In later experiments we showed that cells undergoing apoptosis also exhibit evidence of nitrosative stress, and the use of 1400 W, a drug inhibiting iNOS, increased corneal endothelial cell survival [62].

L-DNase II is also activated in other ocular tissues like the retina. There, it is activated during development, either in the chicken [56], or in the rat (unpublished results) as well as in light-induced retinal degeneration [64].

In cell culture several apoptotic paradigms show activation of L-DNase II. Sometimes it is in parallel with an activation of caspases, like in long-term cultured HeLa cells [55], or in Hepatic F258 cells [61], sometimes independently of caspases like in L1210 [58], or HL-60 cells [32], induced to die by staurosporin. In the last two paradigms, it is interesting to point out that caspases are completely inhibited: in HL-60, because of a pharmacological inhibition, and in L1210 cells because of a point mutation of Apaf-1 that impairs its capacity to stabilize the apoptosome and therefore to activate caspase-9. The same has been found in colon cancer cells [60], strongly suggesting that LEI/L-DNase II could be an alternative pathway when caspases are inhibited, or poorly expressed like in adult neurons.

From the different studied models it comes out that LEI/L-DNase II is activated mostly after metabolic stress and that genotoxic stresses like etoposide or cisplatin are less efficient in activating this pathway. Although this can be generalised to most of the inducers, for some of them the activated pathway depends also on the cell type. So that, staurosporin activates L-DNase II in L1210 cells but not in somatolactotrope cells [85]. Up to now, one single inducer has shown to systematically induce the LEI/L-DNase II pathway. This is HMA (hexamethylene amiloride), a compound that decreases intracellular pH by inhibiting the Na–H exchanger [57].

8. The activation of L-DNase II and the onset of apoptosis

Current data concerning dual activity of LEI/L-DNase II are summarized in Fig. 7. LEI mediates caspase-independent apoptosis in some particular conditions, i.e. when serine proteases having LEI as a target are massively activated. This takes place during metabolic and oxidative stress, when cells are unable to activate caspases, due to terminal differentiation (like in retinal pigmented epithelium cells [59]) or to acquire mutations in cancer cells [58,65]. The apoptotic effect of L-DNase II depends on its endonuclease activity, but also on its nuclear translocation because overexpression of the K225A mutant (impaired for nuclear translocation) decreases the pro-apoptotic activity of L-DNase II as much as the endonuclease-inactivated mutant protein. This effect depends upon the disability of the K225A mutant to be nuclearised, since this mutation does not affect endonuclease activity [73].

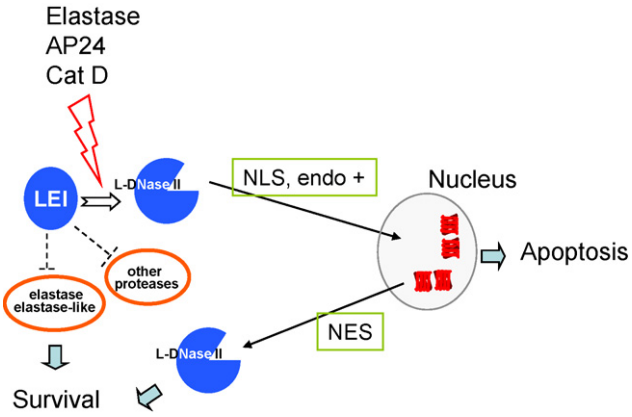

Fig. 7 – Summary of LEI/L-DNase II activation and control. After receiving the appropriate stress, there is an increase of the activity of certain proteases like AP24 and LEI is cleaved. This cleavage exposes the NLS and the endonuclease active site of L-DNase II. L-DNase II is then transported to the nucleus where it digests DNA. In healthy cells the baseline production of L-DNase II can be controlled by the NES of LEI that rapidly extrudes the molecule from the nucleus. In non-stressed cells, the anti-protease activity of LEI will promote cell survival by inhibiting several proteases.

An interesting feature of this molecule is that the cleavage leading to the transformation into L-DNase II is the same as the one involved in the anti-protease function of the molecule, so that, a base line of production of L-DNase II is unavoidable in healthy cells. One means of controlling this inconvenience is to extrude the produced endonuclease from the nucleus, by using the NES as explained before. It is worth to remember that site-directed mutagenesis of the aminoacids of this consensus sequence reduces nuclear export of LEI and induces cell death, confirming that LEI/L-DNase II subcellular localization is determinant for its pro-apoptotic activity. This also points out the existence of mechanisms that may regulate the balance of the NLS on the NES (and *vice versa*) to drive LEI/L-DNase II subcellular localization, depending on the cellular context. Other factors might also be involved in the accessibility of the NES. Preliminary results obtained in our laboratory indicate that this sequence might be phosphorylated a post-translational modification that might impair the nuclear translocation of L-DNase II.

9. LEI/L-DNase II and cross-talk with other molecular regulators of apoptosis

Recent work performed in our laboratory showed that LEI can interact with other effectors of apoptosis. We have shown that LEI overexpression is able to induce poly(ADP-ribose) polymerase 1 (PARP-1) activation (Leprêtre et al., unpublished work). This generates an increase in cytoplasmic poly(ADP-ribose) (PAR) that may trigger apoptosis inducing factor (AIF) release as it has been previously demonstrated [86]. Preliminary data indicate that AIF and LEI may also interact,

suggesting that LEI/L-DNase II may participate to, or boost the PARP–AIF loop to drive cell death.

Native LEI can also interact with caspases and this interaction has anti-apoptotic effects. In etoposide-induced apoptosis, various studies have demonstrated the importance of caspases and recent studies have highlighted the importance of caspase-8, which is activated independently of the death receptor pathway by interchain caspase-8 cleavage. We recently demonstrated that LEI inhibits etoposide-induced apoptosis [103] by inhibiting caspase-8 activation.

It is interesting to note that although LEI is a ubiquitous protein, levels of expression are very variable in different cells and tissues (unpublished results). So that tissues expressing higher levels of LEI may add this mechanism of control of caspases activity to others previously described, like IAPs. So, the level of expression of LEI together with the nature of the cellular injury and the concomitant activation of other pathways may modulate cell survival.

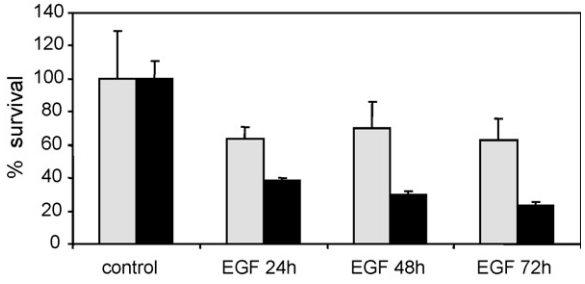

Fig. 8 – LEI/L-DNase II in non-apoptotic cell death. Cells from rat neurohypophysis were induced to differentiate by EGF. During this process, 40–50% of the cells die by a paraptosis-like cell death, with L-DNase II activation. The overexpression of LEI in these cells increases cell death. White bars represent cells transfected with the empty vector and black bars represent cells overexpressing LEI.

10. LEI/L-DNase II and other forms of cell death

We indicate in the precedent paragraph that LEI shows intraction with PAPR and AIF. Although these molecules have been involved in apoptosis, it is currently believed that the PARP–AIF loop is activated during programmed necrosis [87–90]. Indicating that LEI can also participate to the programmed necrotic cells death.

In ARPE-19 cells, a cell line derived from human retinal pigmented epithelium, the exposure to 4% ethanol resulted in a death of 50% of the cells [59]. In order to characterise this cell death, we first have studied DNA degradation. Flow cytometry indicates a sub-G1 DNA population and gel electrophoresis showed the absence of DNA ladders. However, other apoptosis markers, like the degradation of PARP-1, and the inhibition of cell death by cycloheximide suggested an apoptotic-like cell death. The cells presented shrinkage of both cytoplasm and nucleus as well as a blebbing membrane. This suggested that ARPE-19 cells induced to die by ethanol execute a programmed cell death with characteristics of both apoptosis and necrosis.

Western blot experiments performed in this cell death paradigm indicated the activation of L-DNase II. In these experiments we have also noticed an increase in the amount of LEI suggesting a raise in the synthesis of LEI, a feature that was confirmed by RT-PCR. It is interesting to note that this increase in LEI mRNA is a very early event, a 50% increase in mRNA is detected only 10 min after adding ethanol to the cultured layer, and the maximum amount of mRNA is identified 20 min later [59].

In somato-lactotrope GH4C1 cells, epithelial growth factor (EGF) triggers a cell death with striking similarities to paraptosis. First, from a morphological point of view, we observed the presence of large, apparently empty vacuoles [91], and secondly it could be efficiently blocked with AIP-1/Alix, but not Alix-CT. These molecules allow to discriminate between classical (caspase-dependent) apoptosis and paraptosis since the former is selectively blocked by Alix-CT whereas the second is blocked exclusively by Alix [92]. However, in contrast to the original description of paraptosis, the cell death observed in this model does not involve MAP kinases and can be blocked by Bcl-2 overexpression [91]. In contrast to paraptosis, this cell death is characterised by the presence of the caspase-independent internucleosomal DNA fragmentation. The analysis of the pathways involved in EGF-induced internucleosomal DNA fragmentation showed that L-DNaseII is activated [85]. In addition, overexpression of LEI in these cells increase cell death induced by EGF, while staurosporine-induced apoptosis is impaired (Fig. 8).

The data discussed above brought some new evidence on the involvement of L-DNase II in different forms of programmed cell death, and suggested that L-DNase II might not only be a classical apoptosis executioner but might also participate in the execution of additional, non-apoptotic cell death. Interestingly, in GH4C1 cells, staurosporine-induced apoptosis does not involve L-DNase II, in contrast with other cells like L1210 murine leukemia cells [58], or HL-60 [32]. The capacity of staurosporine to induce the activation of L-DNase II pathway appears therefore cell-type specific.

11. LEI/L-DNase II and the serpin superfamily of proteins

Several studies show that serpins of the clade B are important in the regulation of apoptosis. For instance, PAI-2 (serpin B2), which has a genetic association with Bcl-2 [93,94], inhibits TNF-induced apoptosis in HeLa cells. PI-9 (serpin B9), which has a high degree of homology with CrmA, inhibits granzyme B and protects cytotoxic lymphocytes from an accidental release of this enzyme from their own granules [95]. Maspin (serpin B5) is one of the genes targeted by p53 and it is involved in tumor invasion. It is translocated to the internal mitochondrial membrane during apoptosis and it is able to induce apoptosis in some cancer cells [96,97]. Opposite to this effect, SCCA-1/2 (squamous cell carcinoma antigen-1/2) (serpin B3 and B4, respectively) increase the resistance of tumor cells to apoptosis [98,99].

The data discussed before show that LEI (serpin B1) has drastically different effects on apoptosis according to the

inducing stimuli. Recently, phylogenetic studies indicate that serpin B1 and B5, aged of 450 millons years, are the first precursors of the clade B serpins. It is interesting to note that both have pro-apoptotic properties in some circumstances, suggesting that this might be the primary function of these family of proteins [99].

12. General conclusions

The data discussed in this work describe a caspase-independent cell death mechanism activated by serine protease and cathepsins. This system adds complexity to the already complex network of molecular pathways leading to cell death. As discussed by other authors [100,101] this network might have been created during evolution by the incorporation of proteins that introduce a selective advantage. This is why many proteins implicated in apoptosis have also other functions during the cell life. This is also the case of LEI/L-DNase II that can negatively regulate the activation of caspases and other proteases, but can also induce a caspase-independent cell death pathway. It has also been hypothesized that the increasing number of molecules controlling cell death during evolution would have transformed a pure accidental necrotic pathway into a tightly controlled apoptotic pathway [102]. This might explain why some apoptotic effectors like LEI/L-DNase II are also implicated in other forms of cell death.

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