LEI/L-DNase II: interplay between caspase-dependent and independent pathways

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TABLE OF CONTENTS

1. Abstract
2. Apoptosis and caspase activation, are they synonymous?
   2.1. Evidence for caspase independent apoptosis
   2.2. Building up of caspase independent pathway
3. The non caspases proteases involved in apoptosis
   3.1. Calpains
   3.2. Cathepsins
   3.3. Serine Proteases
4. The caspase-independent pathways already characterized: GAAD and LEI/L-DNase II
   4.1. GAAD
   4.2. LEI/L-DNase II
5. LEI and L-DNase II in the living and in the apoptotic cells
6. The cell death redundancy
7. Acknowledgements
8. References

1. ABSTRACT

Caspase activation has been seen, for several years, as the biochemical marker of apoptosis. However, in 2005 the Nomenclature Committee on Cell Death (NCCD) established 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. Actually, the controlled destruction of the cell is coordinated by a proteolytic system involving caspases but also other proteases like cathepsins, calpains and serine proteases. These enzymes participate in an activation cascade that culminates in cleavage of a set of proteins resulting in disassembly of the cell. This disassembling also includes the activation of endonucleases that will destroy a potentially harmful DNA. A caspase-activated DNase performs DNA degradation in caspase-dependent apoptosis, but other endonucleases like L-DNase II or GAAD are activated in caspase-independent apoptosis, allowing the complete dismantling of the cell.

2. APOPTOSIS AND CASPASE ACTIVATION, ARE THEY SYNONYMOUS?

Cell death can be certainly classified according to its morphological features into apoptosis, necrosis, autophagy, mitotic catastrophe, paraptosis etc. Among these different cell deaths, apoptosis is by far the most studied process. The genetic analyses of C. elegans cell death, carried out mostly in the laboratory of H. Robert Horvitz, led to the elucidation of genes that control this cellular suicide mechanism (1-6). Further studies identified caspases as mammalian counterparts of these genes (7, 8). An important issue with respect to apoptosis signalling pathways is the timing of the ‘point-of-no-return’. This event is defined as the step in the signalling process after which termination of the apoptosis-inducing stimuli does not prevent the execution of apoptosis. Elegant studies with nerve growth factor deprivation suggested that the ‘point-of-no-return’ occurs downstream of cytochrome c release and coincides with executioner caspase activation (9, 10).
Caspase-independent apoptosis

From then on caspase activation has been seen as the biochemical marker of apoptosis and a textbook equation was established stating that apoptosis = caspase activation = non-immunogenic cell death (11-14). Although this formula is sometimes applicable, it constitutes an incorrect generalization. Apoptosis can be achieved without caspase activation, and caspase activation does not necessarily cause cell death (15). For example, caspase-9 knockout mice or caspase-3 knockout mice died shortly after birth and had excess of brain tissue, which appeared to be a consequence of defective apoptosis (16-20). However, cell death in other organs was less prominently affected.

Moreover, characterization of *C. elegans ced-3* mutants using light microscopy revealed that in the male gonad, the linker cell undergoes cell death even in the absence of *ced-3* (1). In mammals, genetic studies on cell death induced by BH3-domain-only proteins, such as Bim, Bim and Bad showed that these proteins, that promote caspase activation and apoptosis, can also kill cells independently of Apaf-1 and downstream caspases. Therefore, mouse embryonic fibroblasts invalidated for Apaf-1 die in response to the overexpression of these proteins (21-24). These dying cells display morphological features of apoptosis.

In 2005 the Nomenclature Committee on Cell Death (NCCD) made up of a selected panel of experts decided, after several months of discussion 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 (25).

### 2.1. Evidence for caspase-independent apoptosis

Embryonic fibroblasts from Apaf-1 knockout mice overexpressing BH3-domain-only proteins display apoptotic morphology but no caspase activation was detected suggesting that the cell death pathway induced by BH3-only proteins bifurcates downstream of Bax and Bak, with one branch being caspase-and Apaf-1-dependent and the other not (23). A suggested candidate for this caspase independent cell death is apoptosis-inducing factor (AIF), a mitochondrial oxidoreductase. Histological studies show that embryonic cavitation is blocked in AIF knockout mice (26-29). However, recent data from a mouse (Harlequin) having a proviral insertion that down regulates AIF called ced-3 (30). Neonatal harlequin mice are indistinguishable from wild-type animals, but they die early from gradual neurodegeneration. It was suggested that they suffer from oxidative stress and that AIF protects neurons from oxidative stress. It would be worth to investigate if Harlequin mice have cavitation abnormalities to reconcile these data.

The above presented data, largely discussed in the literature do no investigate the caspase substitute that allows cell death to proceed. As caspases are proteases, it is possible 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-dependent proteases. They are responsible for caspase-independent cell death in breast cancer cells induced by vitamin D (33-35). Cathepsins are lysosomal proteases. Mice lacking cystatin B (endogenous inhibitor of cathepsins B, H, L and S) show cerebellar degeneration (36, 37). Granzyme B, a serine protease, induces cell death mediated by cytotoxic T lymphocytes. Its pro-apoptotic activity can be seen in the presence of caspase inhibitors (38-40).

### 2.2. Building up of caspase independent pathways

During apoptosis, the controlled destruction of the cell is coordinated from within. The central component of this machinery is a proteolytic system involving many proteases. These enzymes participate in an activation cascade that is triggered in response to pro-apoptotic signals and culminates in the cleavage of a set of proteins resulting in disassembly of the cell. This disassembling is achieved by the action of proteases but also of endonucleases that will destroy a potentially harmful DNA.

Nuclease activity is important for the commitment of cells to apoptosis. The inhibition of DNA cleavage delays the appearance of embryonic corpses in *C. elegans* and generates extra cells (41). ICAD-deficient mice are resistant to multiple apoptotic stimuli, and exhibit extra neurons (42-44). Moreover, dysregulation of DNA fragmentation might be directly linked to the induction of autoimmunity (45-47).

### 3. THE NON-CASPASE PROTEASES INVOLVED IN APOPTOSIS

#### 3.1. Calpains

Calpains are calcium sensitive, non-lysosomal cysteine proteases. Two forms of calpains, µ-calpain and m-calpain (also called calpain I and II, respectively), are ubiquitously expressed in human cells. Calcium plays a pivotal role in apoptosis. The release of calcium from the endoplasmic reticulum (ER) triggers mitochondria calcium overload which contributes to loss of mitochondrial membrane potential (MMP) and release of cytochrome c (48). Besides, elevated cytosolic calcium levels activate calcineurin, which dephosphorylates p-Bad and causes its translocation to the mitochondria leading to activation of Bax (49).

Calpains can also be activated by calcium influx. µ- and m-calpains are heterodimers consisting of a distinct large 80-kDa catalytic subunit encoded by the genes *capn1* and *capn2*, respectively, and a common 28-kDa regulatory subunit encoded by *capn4*. The small subunit is indispensable for the activity of both calpains (50).

Recently, these enzymes have been implicated in both pro and anti-apoptotic functions. *capn 4 -/-* 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 (51). Cerebellar granule cells and cortical neurons...
Caspase-independent apoptosis

treated with anandamide were significantly protected by the calpain inhibitors calpastatin and calpeptin (52).

Calpain activation has been associated with several neurodegenerative diseases (53). Alzheimer’s disease (AD) is one of the conditions which calpains have been most commonly associated with. They are thought to be involved in hyperphosphorylation of tau proteins. The proteolytic action of calpains over tau and other neurofilament proteins is related to the necrotic cell death observed in AD. In Huntington’s disease, calpains have been shown to cleave the huntingtin ( htt) protein at multiple sites, generating toxic fragments (54). Overexpression of m-calpain has been detected in brains of patients with Parkinson’s disease, as well as Duchenne and Becker diseases, two neuromuscular disorders (55, 56). Calpains can degrade several substrates involved in cell adhesion, such as focal adhesion kinase (FAK), -catenin and integrins. Moreover, several members of the Bcl-2 family are processed by calpains (57). During trophic factor deprivation in sympathetic neurons, calpains cleave Bax into a pro-apoptotic 18-kD fragment which promotes cell death during apoptosis may, however, extend their life-time (42). Only a very few cellular substrates of calpains have been identified so far. Bid remains the best characterized (69). Although calpains activity is partly regulated by the unfavourable neutral pH in cytosol, their endogenous inhibitors, stefins, cystatins, serpins and thyropins, constitute the major defense mechanism (70). The essential role of inhibitors was confirmed by ablation of stefin B that generates cerebellar apoptosis.

3.3. Serine proteases

Cell death in ER-stressed R6 fibroblasts and in IL-3-deprived monocytes proceeds when caspases are inhibited (71). However, a chymotrypsin-like inhibitor prevents pyknosis, membrane blebbing and phagocytosis. CytC release is blocked by this treatment (72, 73). In ethanol-induced hepatoma cells a trypsin-like inhibitor avoided CytC release (74). More recently Stenson-Cox et al. showed that the pan-caspase inhibitor Z-VAD.FMK did not prevent all apoptotic features activated in staurosporine-treated HL-60 cells, while it abrogated caspase-3 activation and cleavage of PARP-1 (75). 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 in cell death programmes (76). Several serine proteases have been individually implicated in apoptosis: granzymes, Omi, AP24, plasminogen activator. Granzymes are found only in the granules of cytotoxic T lymphocytes and natural killers. Granzyme B can activate multiple components of the apoptotic machinery in target cells by direct activation of caspases and by cleavage of proapoptotic proteins (77). It can also induce features associated with apoptosis through direct cleavage of specific proteins like the kinase Rock II and DFF45. Granzyme A is a highly selective tryptic protease that triggers all of the morphological features associated with apoptosis. However, Granzyme A does not activate caspase family members, nor does it cleave key caspase targets like lamin B or PARP-1 (78). Omi is a mammalian serine protease from the HtrA (High temperature requirement A) family that can be released from the mitochondria. It then binds to IAPs, neutralizing their inhibitory action upon caspases. Recent data from KO mouse support the hypothesis that it essentially acts as a remover of misfolded proteins in the mitochondria (79). Apoptotic protein 24 (AP24) is a 24-kDa serine protease with an elastase-like
activity that triggers oligonucleosomal DNA fragmentation (80, 81).

4. THE CASPASE-INDEPENDENT PATHWAYS ALREADY CHARACTERISED: GAAD AND LEI/L-DNase II

The orderly cell dismantling seen in apoptosis involves, other than proteolysis, chromatin processing at the oligonucleosomal level as first reported by Wyllie (82, 83). Afterwards, DNA laddering has been considered as the molecular hallmark of apoptosis and the search of DNases, e.g. enzymes cleaving DNA, became a major goal in the characterisation of this pathway (42, 84). Several enzymes were proposed as candidates, including DNase I, DNase II, cyclophilins, and DNASE1L3 (85).

None of them, however, appeared to fulfil the criteria for the apoptotic DNase. The existence of several apoptotic pathways activating several nucleases could be hypothesised from works concerning induction of apoptosis in cancer cells. As early as in 1995 it was shown that treatment of murine leukemic L1210 cells with either cisplatin or staurosporine led to apoptosis (86). In contrast, the cell line L1210/DDP was resistant to cisplatin, but staurosporine induced cell death and internucleosomal DNA cleavage. Further work indicated that those stimuli activate different endonucleases (87). However, the importance of this work and others indicating the existence of different apoptotic pathways was neglected due to the increasing importance of the knowledge on caspases activation by that time (88).

The assumption that caspases' activation represents the “point of no return” in apoptotic cascade led to the search of a DNase activated by caspases. In 1998 Nagata's group identified an endonuclease that degrades DNA during apoptosis in mouse and called it CAD, for Caspase-Activated DNase (89-91). CAD is synthesised with its inhibitor, ICAD, a protein that is also responsible for the correct folding of CAD. Actually ICAD seems to function as a chaperone for CAD during its synthesis. The complex ICAD/CAD is localised to the cytoplasm. ICAD inhibits nuclease activity of CAD and also masks its nuclear localization signal. When the cell receives an apoptotic stimulus that activates caspases-3, this protease will cleave ICAD. CAD will then be released and will be able to go to the nucleus and degrade DNA.

Thus, caspases activate CAD by cleaving its inhibitor. If we assume that the other proteases activated during apoptosis may act in the same way, the non-caspase proteases mentioned above should activate, directly or indirectly, other endonucleases. However, the link between these different endonucleases and the non-caspase proteases has not been completely stated, and up to now very few proteases were linked to the activation of endonucleases. As a matter of fact only two endonucleases have been related downstream of proteases activation: GAAD (Granzyme A-activated DNase) and L-DNase II (LEI-derived DNase II). In addition, the release of AIF (Apoptosis Inducing Factor) has also been related to protease activities although its action on DNA is different from the above mentioned enzymes.

4.1. GAAD

Granzyme A (GzmA) and Granzyme B (GzmB) are serine proteases expressed by cytotoxic T cells and Natural Killers. They induce cell death in a perforin-dependent manner (92). GzmA is a serine protease, similar to trypsin but highly substrate-specific (93). Mice KO for GzmA are immunocompetent but they are unable to contain herpes simplex virus neuronal infections.

Cell death induced by GzmA and perforin occurs within minutes after perforin delivery and exhibits all the morphological features of apoptosis. However, there is no activation of caspases. Moreover, cells that are resistant to caspase-mediated cell death, including those that overexpress bcl-2, are sensitive to GzmA (94). Mitochondrial apoptotic mediators like AIF and EndoG or Omi are not released (78).

In 2003 Lieberman's group identified a Granzyme A-activated DNase (GAAD) and its inhibitor (95, 96). By loading cell extracts onto a protein affinity matrix containing a protease-activity mutant of GzmA they isolated a 270-420 kDa protein complex, the SET complex. This complex promoted DNA cleavage in vitro but Granzyme A treatment accelerated the process, indicating that Granzyme A plays a critical role in releasing the endonuclease activity from the complex. By cleaving three of the proteins in the SET complex, GzmA activates NM23-H1, a DNase that makes single-stranded DNA cuts. In addition, GzmA digests the nuclear lamina and the histones. GzmA also disrupts mitochondrial function through unknown mechanisms. The SET complex is associated with the endoplasmic reticulum, and its normal function in the cell is not clearly understood although it was proposed that it is involved in the repair response to oxidative stress and damage (96). In this way, GzmA activates a caspase-independent apoptotic pathway.

4.2. LEI/L-DNase II

The activation of L-DNase II was first discovered in the lens during lens cell differentiation which is an apoptosis-related process (97-98). The activation of this enzyme has also been seen in neural apoptosis during retina development or light-induced retinal degeneration, as well as in apoptosis of other terminally differentiated cells like corneal endothelial cells or in cell culture (76, 81, 88, 99-107).

The key molecule in this pathway is LEI (Leukocyte Elastase Inhibitor), also known as serpinb1 in the new serpin classification. LEI belongs to the Serpin (Serine Protease Inhibitors) superfamily and it can be classified among the ovalbumin serpins which are predominantly intracellular (108-110). Like most serpins, LEI has an anti-protease activity. In its native form, it inhibits elastase, cathepsin G and proteinase 3 (111). But LEI may also be post-translationally modified, either by exposure to an acidic pH or by the action of proteases like elastase (98). Treatment of LEI by these
Caspase-independent apoptosis

Figure 1. The Stressed to relaxed transition. Inhibitory serpins are molecules that fold into a native metastable state (also called stressed state) (RasMol native antithrombin PDB 1E03). Upon docking with a target protease, the reactive center loop (RCL) (arrow head) is cleaved (RasMol cleaved antithrombin PDB 1ATT). The N-terminal region of the site of proteolysis inserts into beta-sheet A (red) (also called relaxed state).

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 endonuclease activity (98). We have called this endonuclease L-DNase II (LEI-derived-DNase II), since it is derived from LEI and shows almost the same pH and ion dependence as DNase II. Hence, this protein has the fascinating capacity of changing its enzymatic activity. During the last few years we tried to elucidate the role of this enzyme in apoptosis. Several questions were raised: what could be the nature of this post-translational modification leading to the conversion of LEI into L-DNase II? What proteins were involved in this conversion? How did the LEI/L-DNase II system work in the living and apoptotic cells?

As stated above, the post-translational modification transforming LEI into L-DNase II reduces the apparent molecular weight of LEI. LEI, like all serpins, is a metastable protein. Its reactive center loop (RCL) (P5-P15 for LEI) is flexible and can adopt different conformations. After cleavage by its target protease at the P1-P1' site, the RCL is incorporated in the main b-sheet (Ab-sheet), thus inducing a loss of the protease-inhibition activity. This mechanism, called the "stressed to relaxed transition", is associated with a change in the apparent molecular weight of the serpin (Figure 1). We have previously shown that cleavage of LEI (42kDa) by elastase results in the appearance of a 35kDa band in denaturing gels (98). This is a mandatory condition for the molecule to display an endonuclease activity.

The analysis of the three-dimensional crystal structure of cleaved equine LEI showed a quite polarized molecule with a higher number of positive charges in the RCL pole, an interesting feature for DNA interaction. Studies performed in our group did not reveal any consensus sequence for endonuclease activity (112). However, it is worth noting the presence of a DH doublet in the active site of endonucleases activated in apoptosis. Analysis of LEI three-dimensional structure shows the presence of two histidines (H287 and H368) in the more positively charged region. From these histidines, we retained the His368 because it is conserved in all species. The introduction of a point mutation on this residue (H368A) results in a molecule that conserves its anti-protease activity but has lost its endonuclease activity (113).

These results indicate that: 1) the insertion of the RCL uncovers a pre-existing endonuclease active site hidden underneath; 2) the endonuclease activity of L-DNase II is related to His368; 3) this activity is blocked in the native conformation because of the steric hindrance of the RCL and 4) both activities, anti-protease and endonuclease, are independent in the molecule. Our site-directed mutagenesis studies also showed that the RCL uncovers a bipartite nuclear localization signal. This signal is exposed together with the endonuclease active site, allowing then nuclear translocation of the cleaved molecule (113).

The transformation of LEI into L-DNase II requires the proteolytic cleavage of the RCL, e. g. the activation of a protease. Among proteases known to cleave LEI, the first is elastase. We found that this enzyme was activated in two models, i.e. in retina pigment epithelium cells induced to die by ethanol and in murine leukemia cells induced to die by staurosporine (104, 105). Other elastase-like proteases activated during apoptosis were also
Caspase-independent apoptosis

Figure 2. LEI/L-DNase II dual function in apoptosis. In living cells, LEI inhibits several proteases promoting cell survival. When the cell is subjected to an apoptotic stress that induces a massive LEI cleavage, the conversion of LEI into L-DNase II is favored. This induces DNA degradation and liberates proteases from inhibition, promoting cell death.

studied, such as, for instance AP24 (apoptotic protease of 24 KDa) (81). This protease is activated during tumor necrosis factor or ultraviolet (UV) light-induced DNA fragmentation in U937 histiocytic lymphoma, as well as during UV light-induced DNA fragmentation in the BT-20 breast carcinoma, HL-60 myelocytic leukemia, and 3T3 fibroblasts (114, 115). The protease was purified by affinity chromatography with DK120, a serine protease inhibitor, and its interaction with LEI was analyzed. We show that L-DNase II is activated in U937 cells treated with TNFα and that L-DNase II activity is suppressed when apoptosis is attenuated with DK120. Moreover, recombinant LEI can interact in vitro with AP24 in a specific SDS-resistant complex. After an extended incubation period, the complex disappears, with concomitant increase in the levels of L-DNase II, indicating that LEI/L-DNase II is the AP24 target that mediates DNA fragmentation during apoptosis in these cells (81).

More recently, we showed that some lysosomal proteases, like Cathepsin D, are able to activate L-DNase II in vitro or in vivo. Cathepsin D is an aspartic protease released from lysosomes in some apoptotic paradigms like etoposide-treated cells or light-induced retinal degeneration (Padrón-Barthe, Chahory, Torriglia, unpublished results).

In addition, other serine proteases than elastase can activate this system. In HL60 cells for instance, staurosporine activates a serine protease-dependent cell death in parallel of caspases. A broad spectrum caspase inhibitor does not affect staurosporine-induced apoptotic morphology, nuclear condensation or DNA fragmentation, despite its prevention of caspase-3 processing. In contrast, this is achieved by inhibitors of chymotrypsin-like serine proteases. Among the inhibited events we found: altered cell morphology, nuclear fragmentation, generation of L-DNase II and DNA degradation.

5. LEI AND L-DNase II IN THE LIVING AND IN THE APOPTOTIC CELLS

L-DNase II activation depends on the apoptotic stimulus received by the cell. Indeed, metabolic stresses are prone to induce LEIs’ transformation into L-DNase II, while genotoxic stresses are not. For instance, LEI/L-DNase II pathway is activated early during apoptosis induced by Hexamethyl Amiloride (HMA), a Na⁺/H⁺ exchanger, while other stimuli, like etoposide, are not able to induce this transformation (103). The L-DNase II pro-apoptotic effect in HMA-induced apoptosis was confirmed by overexpression experiments (103, 113). These experiments also showed that LEI protects cells from etoposide-induced apoptosis (103). In this paradigm LEI is not transformed into L-DNase II and apoptosis is mediated by caspases (116, 117). It seems then that LEI/L-DNase II behaves as a two edges sword: LEI has an anti-apoptotic activity yet L-DNase II has a pro-apoptotic activity.

In etoposide-induced apoptosis, various studies have demonstrated the importance of caspases and recent studies have highlighted the importance of caspase-8 (116, 118). In this paradigm, caspase-8 is activated independently of any death receptor, either by self-oligomerization or by cathepsin D which is released from lysosomes (119). Our recent results indicate that LEI might inhibit cathepsin D protecting the cell against the increase in caspase activity (Padron-Barthe et al, submitted).

It is interesting to note that although LEI is an ubiquitous protein, levels of expression are very variable in different cells and tissues (unpublished data). Thus, tissues expressing higher levels of LEI may add this mechanism for the control of caspases activity to others previously described, like IAPs (120, 121). So that, the level of expression of LEI, together with the nature of the cellular injury, may modulate cell survival.

These results lead us to propose that LEI plays a critical role in apoptosis (Figure 2), acting as a molecular switch between living cells and apoptotic cells. Its double function is to prevent the proteolytic cascade of apoptosis in living cells while the transition to L-DNase releases this proteolytic inhibition and induces nuclear degradation in apoptotic cells.

6. THE CELL DEATH REDUNDANCY

Despite the importance of the discovery of apoptosis as a cell death program indispensable for embryogenesis and protection against uncontrolled cell growth, other less studied pathways to cell death exist (122, 123). Actually, the apoptosis-necrosis paradigm largely argued is too simple to encompass the wide spectrum of possibilities an organism has to eliminate faulty and
potentially dangerous cells. These include autophagy, paraptosis and other forms of cell death (123). This results in the fact that not only caspases, but also calpains, cathepsins, endonucleases, and other proteases can mediate and execute programmed cell death. Moreover, these cell death effectors can be released or activated by several cellular organelles, including mitochondria, lysosomes, and the ER. We have here described several models of caspase-independent cell death but these different routes may overlap and several characteristics of different forms of cell death may be displayed at the same time. The evolutionary advantage of the existence of different death pathways protects the organism against the development of malignant diseases as many barriers have to be beaten before a cell becomes a cancer cell. This may explain the rarity of cancer cells, in view of the number of cell divisions and mutations. The knowledge of caspase-independent pathways is important, as they could be manipulated to develop new cancer therapies.

In addition, increased apoptosis has been suggested to be a feature of many neurodegenerative diseases (124-127). Here the role of caspases seems more limited than elsewhere. For instance, the treatment of neurons with potassium-deprived medium induced activation of caspases. If caspase inhibitors such as Z-VAD-FMK were used to block apoptosis, neurons were going on to die with slowed-down kinetics, but still exhibited morphological features of apoptosis (128-130). Therefore, the use of combined therapies using caspase-dependent and -independent inhibitors in degenerative diseases could improve neural survival and perhaps neural function.

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