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Review

Calcium-dependent and aspartyl proteases in neurodegeneration and ageing in *C. elegans*

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Abstract

Proteolytic mechanisms have been implicated in the process of ageing, and in many neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases, which are most prevalent in old age. Simple model organisms such as the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*, which offer the prowess of sophisticated genetic approaches, have contributed to our understanding of ageing and neurodegeneration. Intensive research in these systems has resulted in detailed models of the ageing process, and also of several neurodegenerative diseases, which recapitulate same aspects of the human pathologies. Inappropriate cell death is a major component of these and other devastating conditions such as stroke. The dissection of the molecular mechanisms underlying the process of cell degeneration in ageing is of outmost importance. Evidence from investigations in *C. elegans* implicates deregulated proteolysis as one major determinant of cellular destruction in neurodegeneration and ageing, and suggests that the process depends critically on the activation of calcium-dependent, calpain proteases and lysosomal aspartyl proteases. Apart from shedding light on important but inadequately understood facets of such phenomena, these discoveries hold promise for developing novel, effective intervention strategies aiming to ameliorate or even counter inappropriate cell death.

Keywords: Acidosis; Alzheimer's disease; Calpain; Cathepsin D; Lysosome; Necrosis

1. Introduction

Enzymatic cleavage of proteins can serve either regulatory purposes, or can result in their catabolic degradation to constituent amino acids. For example, specific proteolytic events are required for the proper maturation, activation or inactivation of proteins. The activity

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of many enzymes and other regulatory proteins is modulated by proteolysis. On the other hand, turnover of unnecessary or damaged protein molecules is achieved through exhaustive protein degradation. Both regulatory and degradation mechanisms play important roles in ageing and in neurodegeneration. In this article, we survey the major proteolytic mechanisms implicated in ageing and neurodegeneration, and introduce *Caenorhabditis elegans* as one organism particularly suited for studies aimed at elucidating these mechanisms. We focus on studies in *C. elegans* that highlight the role of specific proteolytic cascades involving calcium-dependent and aspartyl proteases in ageing and neurodegeneration. We correlate these findings with similar observations in mammals, which suggest that the principal role of protein degradation in ageing and neurodegeneration is conserved across diverse species.

2. Proteolysis and cellular destruction

2.1. Neurodegeneration and proteolysis

Neurodegeneration is an integral component of various human diseases, such as Alzheimer's, Parkinson's and Huntington's disease, amyotrophic lateral sclerosis, and also occurs in pathological situations such as epilepsy, stroke, hypoglycaemia and trauma (Yamashima, 2000). Most of these disorders are frequent in old age. Alzheimer's disease is one of the most common types of dementia in the elderly. It is characterized by the formation of extra-cellular plaques of beta-amyloid peptide (Abeta) and intra-cellular tangles of hyper-phosphorylated tau, a cytoskeletal protein. In Parkinson's disease cytoplasmic inclusions, mainly composed of the pre-synaptic protein α-synuclein, are formed. The inclusions are called Lewy bodies and are found within neuromelanin-containing dopaminergic neurons that project to the caudate/putamen (Maccioni et al., 2001). These neurons progressively degenerate. Finally, the term "polyglutamine diseases" is used to collectively describe disorders such as Huntington's and Kennedy's disease, dentatorubro-pallidoluysian atrophy and six forms of spinocerebellar ataxia. They are caused by self-association and aggregation of molecules with expanded polyglutamine tracts and lead to progressive neurodegeneration and death (Taylor et al., 2002).

Inappropriate cell death underlies the pathology of neurodegenerative pathological conditions. Experiments in primates, rats and mice demonstrate that both apoptosis and necrosis are involved in neurodegeneration. The prevalence of one process over the other mostly depends on the intensity of the insult, neuronal maturity, intra-cellular calcium and ATP levels and downstream signal transducers (Yamashima, 2000). Apoptosis and necrosis represent two morphological extremes of cell death (Majno and Joris, 1995). Apoptosis is a genetically-regulated process, which leads to cytoplasmic shrinkage and karyorhexis. Although it can be executed through distinct mechanisms, apoptosis involves activation of caspases, which are specific cysteine proteases. Caspase-3 is considered to be a key molecule (Hengartner, 2000). Necrosis is more chaotic and results in cytoplasmic swelling and karyolysis or pyknosis (Majno and Joris, 1995). Although necrosis is less studied than apoptosis, experimental evidence in diverse situations suggests the participation of non-specific catabolic proteases, such as acidic lysosomal proteases, in the process (Syntichaki and Tavernarakis, 2002; Yamashima, 2000). In addition to these proteases, calcium-dependent,

calpain proteases have been implicated in cell death. Calpains, which bind calcium, are activated by an increase of intra-cellular calcium levels and subsequently induce lysosomal membrane rupture and leakage of hydrolytic enzymes to the cytoplasm. Calpains also attack cytoskeletal proteins such as spectrin and fodrin, and other structural, cellular components, causing cell collapse (Aki et al., 2002; see Section 5.2 below).

2.2. Ageing and protein degradation

The essential structural and catalytic roles of proteins in the cell are the basis of a long-standing interest in the effects of ageing on protein turnover (reviewed by Ward, 2000). Damage to cellular macromolecules such as proteins has been postulated to be a major contributor to the ageing of diverse organisms. During their functional life, proteins can accumulate damage in the form of several types of deleterious modifications (oxidation, glycation, isomerization, carbonylation, etc.) as a consequence of normal cellular metabolic activity and possibly aberrant circumstances such as exposure to adverse conditions. Damage can be limited by maintaining high anti-oxidant defenses and by clearing/repairing damage efficiently. The process of protein turnover (protein synthesis and protein degradation) constantly replaces damaged/non-functional proteins with new, functional ones. Two major proteolytic pathways contribute to protein turnover; first, the lysosomal system (i.e. cathepsins B, H, L and D); and second, the ubiquitin–proteasome-dependent system (Stryer, 1988). However, changes in the efficiency of protein turnover and also in the rate of protein modification, result in the accumulation of damaged proteins that contribute to progressive decline of cellular function, which accompanies old age.

A combination of increased oxidative damage in old cells and a dramatic decline in protein synthesis and degradation appear to be the major factors responsible for build-up of modified forms of proteins. For example, accumulation of damaged mitochondria, deleted for DNA, may contribute to elevated levels of ROS (Brand, 2000; Melov, 2000; Melov et al., 1994, 2000). Also, an age-related decline in antioxidant defense proteins such as SOD and catalase has been reported in several species (Larsen, 1993; Mo et al., 1995; Oh-Ishi et al., 1995; Rosenberger, 1991). Moreover, the ability of cells to express heat shock proteins involved in protein maturation, transport and stability greatly declines with age (Heydari et al., 1994). A concurrent decline in protein synthesis and degradation (Lee et al., 2000a; Rattan and Clark, 1996) has been suggested to be a major contributor to the accumulation of modified proteins that accompanies ageing (Stadtman, 1988, 1992). This decline in protein turnover has been reported in several organisms ranging from nematodes closely related to *C. elegans* (Prasanna and Lane, 1979; Reznick and Gershon, 1979; Sharma et al., 1979) to mammals (Rattan and Clark, 1996), and can thus be considered a conserved component of a “public” (commonly experience by many organisms) ageing mechanism (Martin et al., 1996). Interestingly, protein turnover rates are maintained at high levels in animals under caloric restriction, and could be instrumental for the beneficial effects of caloric restriction on lifespan and protection against neurodegenerative disorders (Duan and Mattson, 1999; Duan et al., 2003; Yu and Mattson, 1999). Whether changes in protein turnover are a cause or a consequence of ageing is not clear, and this question, while central to the biology of ageing, has not been a focal point of modern ageing research.

3. The nematode *Caenorhabditis elegans*

C. elegans is a small (approximately 1 mm), free-living hermaphroditic nematode that feeds on an *E. coli* diet in the laboratory and completes a reproductive life cycle in 2.5 days at 25 °C, progressing from a fertilized embryo through four larval stages to become an egg-laying adult. Under adverse conditions, such as starvation, over-crowding or high temperature, larvae can enter an alternative life stage called the dauer (enduring) larva, during which animals move but do not feed. The dauer larva is a "non-ageing" organism that survives for weeks or even months (Klass and Hirsh, 1976). When a dauer larva encounters favorable environmental conditions, it re-enters the life cycle at the fourth larval stage, progresses into adulthood to reproduce and then completes the final week or so of its lifespan.

With a relatively short lifespan (a mean value of roughly 13 days at 25.5 °C; Larsen et al., 1995), *C. elegans* offers an attractive platform for the experimental investigation of neurodegeneration and the ageing process (Baumeister and Ge, 2002). The ability of *C. elegans* to reproduce by self-fertilization renders the production and recovery of mutants easy—homozygous mutants segregate as F2 progeny of mutagenized parents without any required genetic crossing. Mutant alleles are readily transferred by mating with males, such that complementation analysis and construction of double mutant strains is straightforward. Positions of thousands of genes on the six *C. elegans* chromosomes have been determined. This genetic map has been aligned with the physical map of the genome (a collection of overlapping DNA clones that spans the six chromosomes). *C. elegans* has a strong foundation in classical genetics and genes can be positioned on the genetic map using standard mapping techniques. The vast majority of *C. elegans* strains studied in research labs are derived from the same parental strain, N2. Thus, in essence, *C. elegans* research involves genetically identical populations, not subject to inbreeding depression (see Gems and Riddle, 2000).

C. elegans development and anatomy are exceptionally well characterized. The complete sequence of cell divisions that occur as the fertilized egg develops into the 959-cell adult has been recorded (Sulston and Horvitz, 1977; Sulston et al., 1983). Serial section electron microscopy has produced a description of the shape and pattern of connection of each of its 302 neurons, so that the full "wiring diagram" of the animal is known (White et al., 1976).

The sophisticated molecular biology of *C. elegans* is greatly facilitated by a completely sequenced genome (The *C. elegans* Sequencing Consortium, 1998). Investigators can take advantage of genome data to perform "reverse genetics" by directly knocking out genes (Liu et al., 1999), or transiently knocking down gene expression by double-stranded RNA-mediated interference, a novel method of generating mutant phenocopies (RNAi; Fire, 1999). In the RNAi protocol, introduced double-stranded RNA corresponding to specific genes can target the homologous endogenous transcript for degradation. In addition, transgenic animals can be created rapidly and with ease (Mello et al., 1991) so that candidate genes involved in ageing can be tested for cell-specific and temporal expression, high copy number expression effects, etc. Vectors are available for cell-specific expression or for generation of reporter fusions to the *E. coli* beta-galactosidase gene (Fire et al., 1990), or the jellyfish green fluorescent protein (GFP; Chalfie et al., 1995). Analysis of ageing tissues is simplified in that all somatic cells are post-mitotic and there is no tissue regeneration.

4. Ageing and neurodegeneration in *C. elegans*

4.1. Ageing

Nematodes exhibit visible changes in behavior and appearance over the course of their lives. As they age, nematodes feed, move and defecate more slowly than their younger counterparts (Bolanowski et al., 1981; Duhon and Johnson, 1995; Klass, 1977). Ageing worms appear rough and lumpy, with a generally distorted morphology. Death is usually assayed by a failure to respond to touch with an eyelash hair, failure to move, and/or failure to pump in food. Old nematodes lose their ability to tolerate various stresses. Old animals are more sensitive to oxidative stress either from higher concentrations of oxygen (Honda et al., 1993) or exposure to oxidizing agents (Darr and Fridovich, 1995; Larsen, 1993), than are their younger counterparts. Similarly, old animals are more sensitive to thermal stress (Lithgow et al., 1995).

C. elegans was the first multicellular organism in which single gene mutations that dramatically extend lifespan had been identified. To date, there are more than 60 known *C. elegans* genes which, when mutated, can extend lifespan. Some of the best studied are the *age*/*daf* mutants that affect an insulin-like signaling pathway required for dauer formation (Friedman and Johnson, 1988a,b; Kenyon et al., 1993), the "clock" (*clk*) mutants in which development and rhythmic behaviors of the nematode are slowed (Hekimi et al., 1998; Lakowski and Hekimi, 1998), mutants with defects in sensory perception (Apfeld and Kenyon, 1999), and the *eat* mutants defective in pharyngeal pumping-thought to experience dietary restriction effects (Hekimi et al., 1998; reviewed by Guarente and Kenyon, 2000). Dietary restriction, administered by *E. coli* rationing or by axenic medium, can also markedly extend *C. elegans* lifespan (Vanfleteren and Braeckman, 1999).

Since some double mutants between members of *age*/*daf* and *clk* exhibit a considerably greater lifespan extension than either single mutant, it has been suggested that *age*/*daf* and *clk* genes participate in two different genetic mechanisms by which lifespan is influenced. Similarly, double mutants of certain *age*/*daf* genes and *eat* genes live longer, suggesting that dietary restriction might act via a different pathway than the *age*/*daf* insulin-signaling pathway.

Interestingly, all tested long-lived mutations of *C. elegans* appear to confer resistance to environmental stress, including oxidative stress, high temperature, and exposure to ultraviolet radiation (Van Voorhies and Ward, 2000; Vanfleteren and Braeckman, 1999). Another feature that seems common to some tested long-lived mutants to date is that metabolic rates of such *C. elegans* mutants are reduced compared with that of wild-type nematodes (Van Voorhies and Ward, 1999). This is mostly true for long-lived *clk-1* mutants but long-lived *age-1* mutants also show an observable reduction of metabolic rates. The *daf-16* genetic suppressor mutations that restore normal longevity to *age-1* long-lived mutants also restore normal metabolic rates (Gems, 1999; Vanfleteren and De Vreese, 1995; Vanfleteren et al., 1998).

4.2. Neurodegeneration

C. elegans is well suited for the study of both normal and aberrant cell death at the cellular, genetic and molecular levels. The animal is essentially transparent throughout

its life cycle and individual nuclei can be readily visualized using differential interference contrast optics. Elucidation of the lineage map has revealed that in certain lineages, particular divisions generate cells which die at specific times and locations and that the identities of these ill-fated cells is invariant from one animal to another (Hedgecock et al., 1983). The ability to easily recognize dying cells within a living animal has allowed easy identification of mutants with aberrant patterns of both apoptotic and necrotic cell death (Chalfie and Wolinsky, 1990; Ellis and Horvitz, 1986; Hedgecock et al., 1983).

C. elegans development includes the programmed death of 131 identified cells (Sulston and Horvitz, 1977; Sulston et al., 1983). Several genes participate in the process (Hengartner, 2000). *ced-3* (*ce*ll *d*eath abnormal) encodes a cysteine protease (caspase) that is essential for death execution. The *ced-4* product activates CED-3 activity and is also required for all programmed cell deaths. In cells fated to live, the death program is held in check by negative regulator CED-9, which can be antagonized by EGL-1. Both activation and negative regulation may be controlled by physical association/multimerizaton of these proteins in the vicinity of the mitochondrial membrane. After death, cell corpses are removed by the products of two groups of genes that act in two parallel pathways (one includes *ced-1*, *ced-6*, and *ced-7*; another includes *ced-2*, *ced-5* and *ced-10*, and *ced-12*). These "undertaker" genes are required for phagocytosis and degradation of dead cells (Hengartner, 2001). Analysis of gene function in *C. elegans* programmed cell death has had an important influence in advancing understanding of mammalian apoptopic death mechanisms because regulators, executors and undertakers of programmed cell death are functionally conserved from nematodes to humans. CED-3 is related to the mammalian caspases that execute apoptotic cell death, CED-4 is related to Apaf-1, CED-9 is a member of the mammalian BCL-2 family and EGL-1 is a member of the death-regulatory BH3-only family (reviewed by Leist and Jaattela, 2001).

Similarly to apoptosis, genetic studies of neurodegeneration in *C. elegans* have greatly facilitated the elucidation of the molecular mechanisms involved (reviewed by Syntichaki and Tavernarakis, 2002). Unusual gain-of-function mutations in several specific *C. elegans* ion channel genes induce necrotic-like deaths of the neurons that express these channel genes. For example, dominant mutations in *deg-1* (*deg*enerin; *deg-1*(*d*)) induce death of a group of interneurons of the nematode posterior touch sensory circuit (Chalfie and Wolinsky, 1990). Similarly, dominant mutations in the *mec-4* gene (*mec*hanosensory; *mec-4*(*d*)) induce degeneration of six touch receptor neurons required for the sensation of gentle touch to the body (Driscoll and Chalfie, 1991). *deg-1* and *mec-4* encode proteins that are very similar in sequence. These genes were the first identified members of the *C. elegans* "degenerin" family, so named because several members can mutate to forms that induce cell degeneration (Chalfie et al., 1993). Included in this family are *mec-10*, which can be engineered to encode toxic degeneration-inducing substitutions, *unc-8*, which can mutate to a semi-dominant form that induces swelling and dysfunction of ventral nerve cord and *unc-105*, which appears to be expressed in muscle and can mutate to a semi-dominant form that induces muscle hypercontraction (Tavernarakis and Driscoll, 2001b). Thus, a general feature of the degenerin gene family is that specific gain-of-function mutations have deleterious consequences for the cells in which they are expressed (Tavernarakis and Driscoll, 2001a). *C. elegans* degenerins share sequence similarity with subunits of the vertebrate amyloride-sensitive epithelial Na^+ channel (*ENaC*) that mediates Na^+ absorption

in epithelia of the distal part of the kidney tubule, the urinary bladder, the distal colon and the lung (Canessa et al., 1993). Because many *C. elegans* degenerins can mutate to toxic forms that induce neurodegeneration, the neuronally expressed mammalian family members are logical candidates for genes that can mutate to cause neurodegeneration in higher organisms. In this regard, it is interesting that mammalian MDEG, engineered to encode an amino acid substitution analogous to the change in *mec-4*(*d*) (see below), induces degeneration when expressed in *Xenopus* oocytes and embryonic hamster kidney cells (Champigny et al., 1998).

Although *mec-4*(*d*) and *deg-1*(*d*) mutations kill different groups of neurons, the morphological features of cell deaths they induce are the same. The time course of degeneration depends upon the dosage of the toxic allele, but on average can take approximately 8 h. When viewed using the light microscope, the nucleus and cell body of the affected cell first appear distorted and then the cell swells to several times its normal cell diameter (Hall et al., 1997). Eventually, the swollen cell disappears, often after shrinking but sometimes as a consequence of cell lysis. Interestingly, the swollen character of *mec-4*(*d*)- and *deg-1*(*d*)-induced deaths resembles the morphologies of mammalian cells undergoing necrotic cell death. At the ultrastructural level, cells dying as a consequence of *mec-4*(*d*) and *deg-1*(*d*) expression exhibit some remarkable features. The first detectable abnormality apparent in an ill-fated cell is the formation of small tightly wrapped membrane whorls that seem to originate at the plasma membrane (Hall et al., 1997). These whorls are internalized and appear to coalesce into large electron-dense membranous structures. Large internal vacuoles form and distortion of the nucleus by these vacuoles is associated with chromatin clumping. Finally, organelles and cytoplasmic contents are degraded, usually leaving a membrane-enclosed shell. The striking membranous inclusions suggest that intra-cellular trafficking may contribute to degeneration. Interestingly, in some mammalian degenerative conditions such as neuronal ceroid lipofuscinosis (Batten disease; the *mnd* mouse; Cooper et al., 1999) and that occurring in the *wobbler* mouse (Blondet et al., 2002), cells develop vacuoles and whorls (fingerprint bodies) that look similar to internalized structures in dying *C. elegans* neurons. This suggests that some degenerative processes may be similar in nematodes and mammals.

mec-4(*d*) and *deg-1*(*d*) alleles encode substitutions for a conserved alanine that is positioned extra-cellularly, adjacent to pore-lining membrane-spanning domain. The size of the amino acid side-chain at this position is correlated with toxicity—substitution of a small side-chain amino acid does not induce degeneration, whereas replacement of the Ala with a large side-chain amino acid is toxic (Driscoll and Chalfie, 1991). Steric interference conferred by a bulky amino acid side-chain prevents such an approach, causing the channel to close less effectively. Increased cation influx initiates neurodegeneration. That ion influx is critical for degeneration is supported by the fact that amino acid substitutions that disrupt the channel conducting pore can prevent neurodegeneration when present in cis to the A713 substitution. In addition, large side-chain substitutions at the analogous position in some neuronally expressed mammalian superfamily members do markedly increase channel conductance. Genetic mosaic analyses first indicated that *mec-4*(*d*) kills as a consequence of a toxic activity within the cells that die. Ectopic expression of *mec-4*(*d*) can induce swelling and death of cells other than the touch receptor neurons, confirming the cell autonomy of *mec-4*(*d*) action (Harbinder et al., 1997). The execution of degenerative cell death occurs by a mechanism that appears distinct from that utilized in programmed cell death (Syntichaki

et al., 2002). At the genetic level, it has been demonstrated that *ced-3*(*lf*) and *ced-4*(*lf*) mutations do not block *mec-4*(*d*)- and *deg-1*(*d*)-induced cell degeneration. Likewise, *mec-4*(*d*) and *deg-1*(*d*) alleles do not disrupt programmed cell deaths.

In addition to degenerins, at least three distinct genes cause morphologically similar cell death in *C. elegans*. First, mutations in additional channel genes such as *deg-3* that increase channel activity, cause vacuolar degeneration of *C. elegans* neurons (Treinin and Chalfie, 1995). *deg-3* encodes a protein related to the vertebrate nicotinic acetylcholine receptor that participates in the formation of a channel highly permeable to Ca^{2+}. Second, expression of constitutively active, GTPase-defective, heterotrimeric G protein $Galpha_s$ (either from *C. elegans* or from rat) causes swelling and degeneration of many (but not all) cells in which the mutant gene is expressed (Berger et al., 1998; Korswagen et al., 1997). Third, expression of the toxic human beta amyloid peptide 1–42, derived from the APP precursor protein and implicated in the pathogenesis of Alzheimer's disease, in *C. elegans* body wall muscles causes animals to become progressively paralyzed as they develop and induces necrotic-like death of some cells around the nerve ring (Link, 2001). Although these genes normally are involved in distinct processes, it remains possible that they share a common death-activating mechanism: alteration of channel activity. Consistent with this possibility, G proteins are known to modulate channel activity. Likewise, some studies have linked beta-amyloid toxicity with altered channel function.

Apart from the genetic origins, necrotic death can be inflicted by hypoxic conditions in *C. elegans*. Interestingly, specific mutations in the gene *daf-2*—a member of the *age*/*daf* group of genes that can mutate to increase lifespan—protect against hypoxic death (Scott et al., 2002).

5. Calcium-regulated proteases

5.1. Calcium homeostasis

Calcium ions participate in a large set of cellular processes. The intra-cellular calcium homeostasis is maintained by a variety of channels and ion pumps, which function either to transport calcium into the cytoplasm or to remove it. Channels that contribute mostly to the increase of cytoplasmic calcium concentration are plasma membrane voltage-, receptor- and store-gated channels, together with the endoplasmic reticulum (ER) inositol triphosphate receptor (InsP3Rs) and the ryanodine receptor (RyR) channels. The removal of calcium from the cytoplasm is carried out mainly by the Ca^{2+}-ATPase pump and Na^{+}/Ca^{2+} exchanger at the plasma membrane, and the sarco-ER ATPase (SERCA) at the endoplasmic reticulum. Na^{+}/Ca^{2+} exchangers and the permeability transition pore in mitochondria also play a role in the process of intra-cellular calcium homeostasis (Berridge et al., 2000).

When normal calcium levels in the cytoplasm are exceeded and homeostatic mechanisms fail to compensate for this anomaly, the consequences to the cell can be devastating. Cell death by either apoptosis or necrosis will commence, depending on the magnitude of calcium overload and on additional accompanying conditions. For example, a higher than normal concentration of calcium ions compromises the mitochondrial membrane, which is followed by cytochrome C release and down-regulation of the apoptotic inhibitor Bcl2, which induces

apoptosis. Alternatively, excessive calcium influx invokes necrosis by causing cell swelling, reduction of plasma membrane integrity, and mal-activation of a proteolytic cascade that dismantles the cell (Carafoli, 2002).

Recent studies in *C. elegans* demonstrate that calcium is a central factor in neurodegeneration. Experiments have revealed at least four proteins of the endoplasmic reticulum that regulate intra-cellular calcium levels and are required for necrotic cell death. These are calreticulin and calnexin, which are calcium-binding chaperones, and the InsP3R and RyR calcium channels (Xu et al., 2001). In addition, gain of function mutations in DEG-3, which is a subunit of the acetylcholine receptor calcium channel induce necrosis in nematodes (Treinin and Chalfie, 1995). In mammals, excitotoxic death, which resembles necrosis in nematodes, is caused by excess release of glutamate and subsequent over-activation of kainate, AMPA and NMDA glutamate receptor channels on post-synaptic membranes (Choi, 1992). Massive calcium entry can occur due to over-stimulation of NMDA receptors, thus triggering cell death.

Disturbance of calcium homeostasis has also been related to the ageing process by experiments with high-density oligonucleotide arrays encompassing more than 11,000 genes, aiming to identify changes in gene expression in the hypothalamus and cortex from young and adult mice (Jiang et al., 2001). Some of the changes observed were common in the two regions, while others were unique to only one of them. Among the genes, whose expression was reduced in both hypothalamus and cortex, were Na^+/K^+-, Ca^{2+}- and H^+-ATPases. These pumps play important roles in the regulation of intra-cellular calcium concentration.

The mechanisms by which calcium induces neurodegeneration are under investigation. A major pathway which has been associated with the process is the activation of calcium-dependent proteases such as calpains.

5.2. Calpains

Calpains are intra-cellular, calcium-dependent cysteine proteases. They are mainly cytoplasmic, although they also act in the nucleus. Two isoforms of calpains have been distinguished, μ-calpain or calpain 1 and m-calpain or calpain 2, activated by micro- and milli-molar calcium levels respectively (Sorimachi et al., 1997. Both are heterodimers, able to bind calcium. μ-calpain is found in dendrites and can be autolytically activated at the plasma membrane. Thus, it contributes to several signaling cascades. Generally, calpains are activated by calcium and inhibited by an endogenous inhibitor, calpastatin. Their function also depends on the state of their substrates. Calpains have been implicated in a wide range of processes, such as development of the nervous system, excitotoxicity, ischemia and hypoxia to the brain, heart and kidney, trauma, environmental toxins effects, allergic encephalomyelitis, behavioral disorders, respiratory diseases, breast cancer and in other pathological cases (Huang and Wang, 2001; Lipton, 1999; Vanderklish and Bahr, 2000). They have been also implicated in ageing processes and in age-related neurodegenerative diseases (Chondrogianni et al., 2002; Nixon, 2000).

Calpain activation occurs in adult-onset neurodegenerative diseases, such as Alzheimer's (Haug et al., 1996; Karlsson et al., 1995; Nilsson et al., 1990) and Parkinson's disease (Mouatt-Prigent et al., 1996). Interestingly, increased μ-calpain activation and reduction of calpastatin levels is also observed in mice deficient for the Klotho protein (*klotho* mice;

Manya et al., 2002), which exhibit prematurely phenotypes of human aging, such as arteriosclerosis, osteoporosis, short life span and infertility. It is suggested that age-related abnormalities in calcium homeostasis and in other cellular processes (e.g. lysosomal dysfunction) contribute to enhanced calpain function. The up-regulation of cellular proteolytic mechanisms, may be also attributed to an increase in the levels of abnormal protein levels, such as those modified by reactive oxygen species (ROS) (Adamec et al., 2000).

Calpains cleave various substrates in vitro and in vivo, such as cytoskeletal proteins, growth factor receptors and transcription and cell cycle-related proteins (Fig. 1). A well-studied substrate is the cytoskeletal protein spectrin (Martin et al., 1995; Masliah et al.,

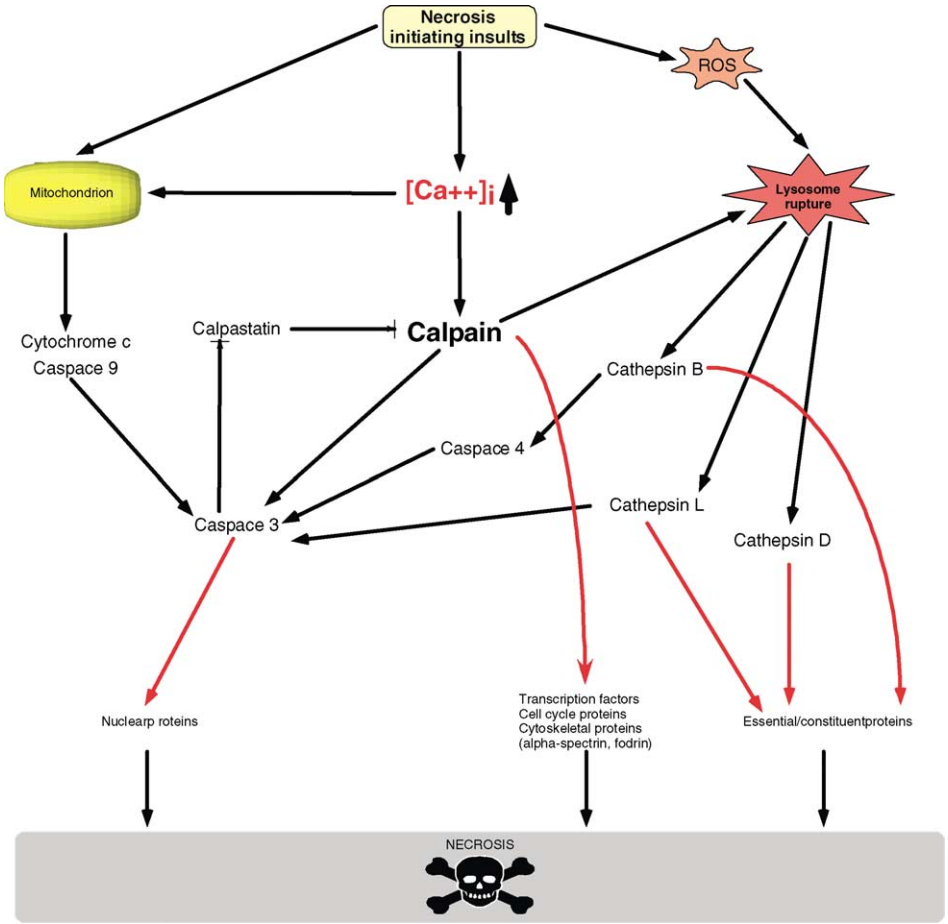

Fig. 1. Proteolytic cascades in necrosis. Calpain has a central role in sensing excessive calcium levels and in turn initiating the death response by activating several classes of effector proteases such as cathepsins. In addition, calpain proteases contribute directly to cell destruction by degrading essential structural proteins. Hence, calpain is both a regulator and an executioner of cell death.

1990). Calpain cleaves spectrin during both necrosis and apoptosis. Cleavage of spectrin produces two stable products, which can be easily detected using specific antibodies. This property is extensively used to study the spatiotemporal pattern of calpain activation. Cain/cabin 1 as well as neurofilaments and fodrin, which are cytoskeletal components, constitute some other molecules also cleaved by calpain (Aki et al., 2002; Kim et al., 2002; Stys and Jiang, 2002). Irregular degradation of these substrates has been associated with different kinds of cell death. Cain/cabin 1 was isolated in a screen for putative calpain substrates. Calpain cleaves the cain/cabin 1 at the C-terminal region. This region contains a calcineurin-binding domain and is able to form complexes with calcineurin. Based on these observations, researchers suggested that one of the mechanisms of calcium-triggered cell death might be the cleavage of cain/cabin 1 by activated calpains. When cleaved, cain/cabin 1 can no longer bind and inhibit calcineurin. Thus, calcineurin is activated and mediates cell death (Kim et al., 2002).

Deprivation of oxygen and/or glucose leads to the breakdown of neurofilaments, which are essential cytoskeletal proteins of neuronal axons, thus causing neuronal damage. In vitro oxygen–glucose deprivation during anoxia and ischemia in isolated rat optic nerves caused degradation of neurofilaments (NFs) 160 and 200, dephosphorylation of the heaviest isoform (NF200) and reduced neuronal function, as assessed by electrophysiological studies. Removal of extra-cellular calcium, blockade of voltage-gated Na^+ channels with TTX, or inhibition of calpains differentially diminished these effects. Thus, calcium overload during oxygen and/or glucose deprivation activates several calpain-mediated pathways, which lead to neural destruction (Stys and Jiang, 2002). Fodrin proteolysis was also observed during necrotic cell death, induced by oxygen deprivation in rat cardiomyocytes (Aki et al., 2002). Hypoxia causes acidosis, which activates Na^+/H^+ exchangers, while the subsequent Na^+ influx might activate Ca^{2+} influx through Na^+/Ca^{2+}-exchangers. Elevation of intra-cellular calcium, derived from extra-cellular and ER-stores, leads to calpain activation, which degrades fodrin and ultimately leads to necrotic cell death.

The role of calpain proteases in neurodegenerative disorders is also of great importance. Neurofibrillary tangles, composed primarily of hyperphosphorylated tau, are a major pathological feature of Alzheimer's disease. Calpain may contribute to the formation of these structures directly by degrading tau or early stage fibrils (Grynspan et al., 1997). Alternatively, calpain proteases may indirectly induce the conversion of normal tau to hyperphosphorylated forms, through activation of PKC kinase (Sato et al., 1997; Shea et al., 1996), or cleavage of p35 to p25. p25 causes prolonged activation and inappropriate localization of cdk5, which leads to tau hyperphosphorylation, cytoskeletal disruption and apoptosis (Lee et al., 2000b). Another feature of Alzheimer's is the deposition of amyloid-β peptide aggregates in the extra-cellular space, forming senile plagues. It is possible that these deposits cause apoptosis through calpain or caspases (Chan and Mattson, 1999; Martin et al., 1995). Caspases promote calpain activation by cleaving calpastatin. Moreover, calpains may activate pro-death caspases (McCollum et al., 2002). This function might be indirect, through calpain cleavage of caspase-8, caspase-9 and APAF-1. m-calpain, that is up regulated in AD and Parkinson's, has also been shown to activate caspase-3 (Grynspan et al., 1997; Mouatt-Prigent et al., 1996), although there is also evidence of caspase-independent contribution of calpains to apoptotic events.

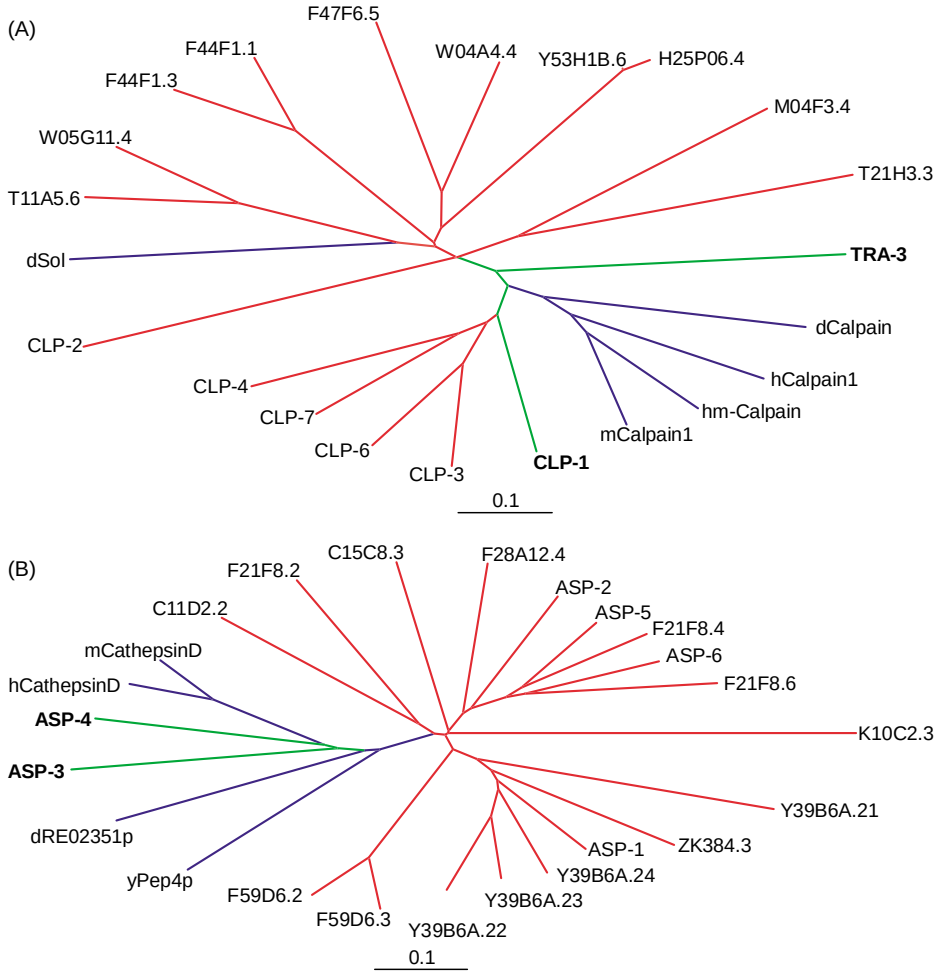

Fig. 2. Calpain and aspartyl protease families of *C. elegans*. (A) Phylogenetic relationships among aspartyl proteases. ASP-3 and ASP-4, shown in bold, are required for neurodegeneration and cluster within a distinct branch that also includes mammalian proteases. Red branches indicate nematode proteins (green for ASP-3 and ASP-4). Non-nematode proteins are in blue. (B) Phylogenetic relationships among calpain proteases. CLP-1 and TRA-3, shown in bold, are involved in neurodegeneration. Red branches indicate nematode proteins (green for CLP-1 and TRA-3). Non-nematode proteins are in blue. Branch lengths represent the expected number of amino acid replacements per site (0.1 in the scale bar).

Calpain activation is required for the execution of neurodegenerative cell death in *C. elegans* (Syntichaki et al., 2002). Reduction of calpain function, by using the specific inhibitor MDL-28170, suppressed necrosis. RNA interference (RNAi) experiments and observations with double mutant strains showed that *clp-1* and *tra-3*, which encode two different calpain proteases of the nematode (Sokol and Kuwabara, 2000; see also Fig. 2A), are mainly involved in cell death process (Fig. 3).

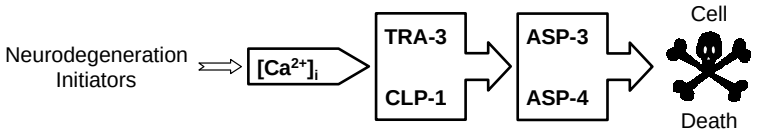

Fig. 3. Calpains and aspartyl proteases are required for neurodegeneration in *C. elegans*. Two specific calpain proteases CLP-1 and TRA-3 function in parallel and upstream of two aspartyl proteases, ASP-3 and ASP-4 to mediate cell death. This arrangement is consistent with a model where calpains act as sensors of elevated calcium concentration and subsequently activate executioner aspartyl proteases that dismantle the cell.

6. Catabolic proteases

6.1. The lysosomal system

Aspartyl proteases are hydrolytic enzymes that perform intra- and extra-cellular degradation of proteins. This class of enzymes includes chymosins, pepsins, renins and cathepsins D and E. All of them contain the highly conserved motif D T/S G T/S at the active site. Cathepsin D is a carboxyl protease, sensitive to pepstatin with an optimum pH of 3, in vitro. It is produced as an inactive pro-enzyme and serially processed to two active forms, a single and a double chain. The conversion from the single to the double chain takes place in lysosomes and requires the function of cysteine proteases or the autocatalytic activity of cathepsin D (Bi et al., 2000b). Studies in nematodes and mammals demonstrate the implication of cathepsin D in ageing and age-related neurodegenerative diseases.

The requirement of aspartyl proteases in neurodegeneration has been recently demonstrated by experiments with nematodes. Reduction of aspartyl protease activity by specific mutations, chemicals or starvation caused reduction of necrosis in *C. elegans* motor neurons. At least six aspartyl proteases (ASP-1 to ASP-6) are encoded in the *C. elegans* genome (Tcherepanova et al., 2000; see also Fig. 2B). Complementation assays and RNAi experiments revealed that necrotic cell death is mediated by the enzymes ASP-3 and ASP-4, which can induce necrosis when overexpressed (Fig. 3; Syntichaki et al., 2002).

Experiments with rats, mice and cultured cells, as well as observations in humans indicate cathepsin D activation in specific aged brain regions and cells. For example, the entorhinal cortex and CA1 hippocampal neurons, which exhibit greater susceptibility in age-related neurodegenerative diseases; CA1 hippocampal neurons degenerate in patients with Alzheimer's disease. A significant age-dependent increase in cathepsin D activity has also been observed in the guinea pig cerebellum neurons and in cerebellum and neocortex neurons of aged mice (Vohra et al., 2002). Cathepsin D activation is also obvious early during neurodegeneration, before neurons exhibit any phenotypic abnormality (Adamec et al., 2000). By using chemical agents to induce apoptotic and/or necrotic cell death in rat primary hippocampal cultures and monitoring cathepsin D activation, Adamec et al. (2000) observed that the lysosomal–endosomal system is up-regulated, with the number and size of lysosomes increasing during slow progressing apoptosis and necrosis. Similar up-regulation is seen during ageing and Alzheimer's disease; lysosomal proliferation has been observed in neurons of aged rats and humans and in vulnerable neurons in Alzheimer's disease (Crawford et al., 2000).

The integrity of the lysosomal membrane declines with age, causing the leakage of hydrolytic enzymes, such as cathepsin D and β-glycuronidase, to the cytocol (Chondrogianni et al., 2002). Cell culture-based assays reveal increased activity of these enzymes in the cytoplasm of aged cells, as well as decreased cathepsin activity in lysosomes. Studies in old rats associate cytoplasmic cathepsin D with neurodegeneration, whereas reduction of lysosomal membrane stability and an increase in cytocolic cathepsin D is observed in aged brain and in Alzheimer's disease. Release of lysosomal enzymes, due to lysosome injury or rupture, has been demonstrated in cases of heart and brain ischemia (Decker et al., 1980). Treatment of primate neurons exposed to ischemic insults with cathepsin inhibitors protects neurons from delayed degeneration (Yoshida et al., 2002). Cathepsin D that leaks to the cytoplasm operates at neutral pH and cleaves the microtubule cross-linking protein tau, possibly leading to the break down of the cytoskeleton. It may also contribute to the generation of phosphorylated tau, which forms neurofibrillary tangles (Bi et al., 2000a).

In contrast with observations in mammals, ageing studies in *C. elegans* have revealed a decline in cathepsin D activity with age (Sarkis et al., 1988). In addition to cathepsin D, the function of cathepsins Ce1 and Ce2 is also reduced in aged worms. Cathepsin D declines about 10-fold from day 3 (early adulthood) to day 11 (near the mean lifespan). The specific activity of thiol protease cathepsin Ce1 declines about 2.5-fold over the same period, and the specific activity of thiol protease cathepsin Ce2 declines about eight-fold (Sarkis et al., 1988). However, in these studies protease activity was averaged over all nematode tissues and therefore no conclusions can be drawn as to what happens specifically in the nervous system. Nevertheless, this reduction is consistent with reduced overall protein turnover late in life (Tavernarakis and Driscoll, 2002). Proteosomal activity also decreases with age. Reduction of protein degradation efficiency in old animals could precipitate the accumulation of oxidized protein products, which tend to form insoluble and non-degraded aggregates intra- or extra-cellularly. Such features have been observed in several age related diseases. Animals exposed to caloric restricted conditions, which prolong life span, exhibit lower levels of oxidized proteins, the decline in the proteosomal activity is attenuated while the activity of proteases like cathepsin D is restored (Merker et al., 2001). Animals are also more resistant to oxidative stress-induced damage, as anti-oxidant defense enzymes are up-regulated. Finally, age associated disorders are delayed.

6.2. The calpain–cathepsin hypothesis

Based on investigations in primates and rats, Yamashima (2000) proposed a mechanism of cellular destruction during necrosis which involves both calpain and aspartyl protease activation, known as "calpain–cathepsin hypothesis". According to this scenario, necrotic insults (such as cerebral ischemia) cause excessive calcium overload of the cytoplasm. The increased calcium levels activate calpains, which induce lysosomal rupture and release of hydrolytic proteases, leading to cell death (Fig. 4). There are several observations in support of this hypothesis. Hypoxia and hypoglycemia in hippocampal slices as well as hyperactivation of NMDA receptors cause elevation of intra-cellular calcium levels. Whole brain complete ischemia in monkeys results in CA1-specific μ-calpain activation, which is localized at the vacuolated or disrupted lysosomal membrane. Additionally, extra-lysosomal leakage of cathepsin B was observed in CA1 neurons about 3 days after whole brain ischemia

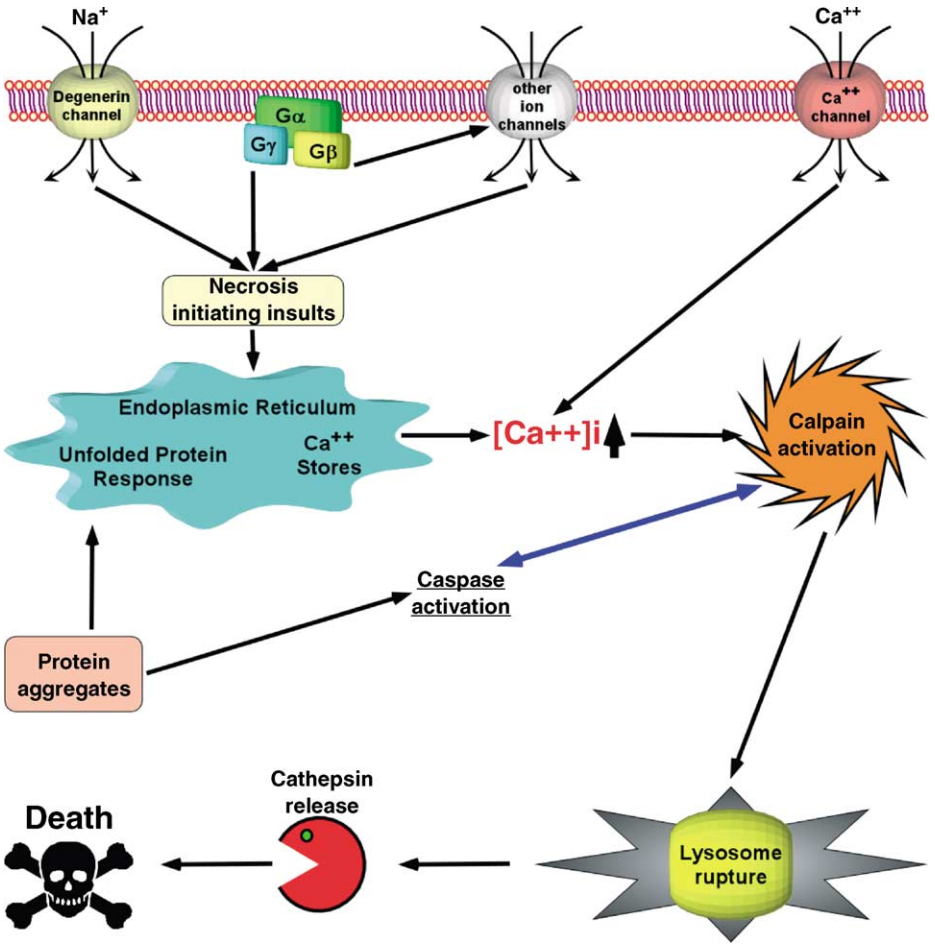

Fig. 4. Necrotic cell death pathways. Various necrosis-initiating insults converge to increase intra-cellular calcium concentration beyond tolerable levels, The increase is a result of calcium influx from extra-cellular space or calcium release from intra-cellular stores (i.e. mitochondria and endoplasmic reticulum), or both. Increased calcium concentration activates calpain, which causes rupture of lysosomal membrane and release of hydrolytic enzymes.

in monkeys. Disruption of lysosomal membrane and leakage of hydrolytic enzymes, such as cathepsins B and L, to the cytoplasm resulting in the necrotic cell death mostly of the hippocampal CA1 neurons has been demonstrated in monkey brains (Yoshida et al., 2002).

The calpain–cathepsin hypothesis is consistent with recent findings on the mechanism of neurodegeneration in *C. elegans*. Elevated calcium concentration in the cytoplasm can be achieved by treating animals with thapsigargin, which induces release of calcium from the ER to the cytoplasm (Takemura et al., 1989), or by gain of function mutations in the acetylcholine receptor calcium channel subunit DEG-3 (Treinin and Chalfie, 1995). Both conditions initiate necrotic cell death in the nematode which can be ameliorated by a

reduction in the activity of calpains CLP-1 and TRA-3 and aspartyl proteases ASP-3 and ASP-4 (Syntichaki et al., 2002).

7. Conclusions and perspectives

Uncommanded cell destruction is central to ageing and neurodegeneration. Proteolytic mechanisms play a principal role in the process of cell death. Two major classes or proteases emerge as key players, which mediate the dismantling of the cell; calcium-regulated calpains and acidic aspartyl proteases. Calpains provide a link between deregulated calcium homeostasis, which signals cellular demise, and downstream non-specific degradation machinery such as lysosomal hydrolases, including aspartyl proteases. This general scheme is likely operational in cases of inappropriate cell death observed in organisms as diverse as nematodes and primates (Syntichaki and Tavernarakis, 2002). As such, it reflects basic common aspects of cellular physiology, which under extreme circumstances are transformed to catastrophic and exterminate the cell. Otherwise, under normal conditions, calpain and aspartyl proteases function to mediate essential signaling and metabolic processes. These proteolytic activities did not evolve specifically to carry out necrotic cell death, unlike caspases, the executioners of apoptotic cell death. The spatiotemporal deregulation that occurs when cellular defenses and homeostatic mechanisms are overwhelmed, allows these enzymes to wreck havoc and express their deadly potential.

Neurodegeneration is a main feature of the most common types of age-related human pathological conditions. Neuronal destruction is accomplished through either apoptotic or necrotic cascades, but also dying cells exhibiting mixed or intermediate features have frequently been observed. Necrotic cell death represents a significant problem in human health. Apart from many neurodegenerative disorders with a necrosis component, excitotoxic neuronal cell death that accompanies oxygen deprivation associated with stroke is a major contributor to death and disability (Lee et al., 1999). Additionally, ischemic diseases of the heart, kidney and brain have been cited as the primary causes of mortality and morbidity in industrialized nations (Epstein, 1986).

Studies in lower organisms, such as in the nematode *C. elegans*, have provided significant insight into the mechanism underlying this cell death process. The development of nematode and fly models that faithfully reproduce features of necrosis in mammals has facilitated the infusion of both forward and reverse genetics approaches into our efforts to obtain a detailed description of the molecular events underlying death. The strong arsenal of molecular biology and biochemical tools available in *C. elegans* and *Drosophila*, coupled with their completely sequenced and highly annotated genomes should greatly accelerate research on necrotic mechanisms. Indeed, many modern, high-throughput, screening procedures such as whole-genome microarray analysis (Kim et al., 2001) and systematic gene knockdown using RNAi (Kamath et al., 2003), allow comprehensive, genome-encompassing searches for genes involved in necrosis. This strategy has already started to pay off and promises even more discoveries in the near future. Dissecting the molecular pathways that lead to neuronal demise is of great importance, as this will open the door to develop new diagnostic methods and to discover novel and more effective therapeutic approaches. The prospect of deploying large-scale screening procedures makes worms particularly promising for the identification

of new targets and the discovery of potential pharmaceutical compounds with applications in preventing or counteracting age-related neurodegenerative pathologies in humans.

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