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Autophagy – a basic mechanism and a potential role for neurodegeneration

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Abstract

Autophagy constitutes a fundamental survival strategy of cells; its disturbance contributes to the pathogenesis of cancer, liver and immune disease, pathogen infection, myopathies as well as neurodegenerative disorders such as Amyotrophic lateral sclerosis, Parkinson`s, Huntington`s and Alzheimer`s disease. The pathogenesis of neurodegenerative diseases also involves a gradual and progressive loss of neuronal cells. Cells may use different pathways for active self-destruction as reflected by different morphology: while in apoptosis (or "type I") nuclear fragmentation associated with cytoplasmic condensation but preservation of organelles is predominant, autophagic degradation of the cytoplasmic structures preceding nuclear collapse is a characteristic of a second type of programmed cell death (PCD). Linking autophagy to programmed cell death initiated a controversial discussion on how a suggested role of autophagy in cell suicide might meet with its established survival function. To some extent, the diverse morphologies can be associated with distinct biochemical and molecular events [caspase-dependent and -independent death programs, DAP-kinase activity, Ras-expression, induction of autophagy genes, fate of cytoskeleton, among others]. However, there is a broad overlap between cell death pathways. Conceivably, diverse PCD programs emerged during evolution, the conservation of which allows eukaryotic cells a flexible response to physiological or pathological demands.

Key words: *apoptosis, necrosis, autophagic cell death, caspase-dependent/-independent cell death, Atg5, Atg7, beclin-1.*

Introduction

Autophagy, in eukaryotic cells, constitutes a degradative mechanism for removal and turnover of bulk cytoplasmic constituents via the endosomal-lysosomal system. Early studies revealed autophagy as an adaptive response of cells to nutrient deprivation, i.e. to ensure minimal housekeeping functions (nutrient recycling). More recently it is recognized that the function of autophagy, in multicellular organisms, is much more complex as it is involved in physiological processes as diverse as biosynthesis (cvt pathway), regulation of metabolism through elimination of specific enzymes,

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morphogenesis, cellular differentiation, tissue remodelling, aging and cellular defence, among others [25,33,37,55,62,77,96,108]. In instances of cell injury or accumulation of neurotoxic aggregates, damaged organelles/membranes or intracellular inclusions may be transferred to the autophagic pathway, serving as the homeostatic mechanism at the subcellular scale [13,31,62,56,60,96,113]. Overall, autophagy constitutes a fundamental survival strategy of cells. On the other hand, autophagy has been also linked to programmed cell death (PCD), initiating a controversial discussion on how a suggested role of autophagy in cell suicide might meet with its survival function [13-15,31,56,60,62,77,96,113].

Disturbance of autophagy contributes to the pathogenesis of cancer, liver and immune disease, pathogen infection, myopathies as well as neurodegenerative disorders such as Amyotrophic lateral sclerosis, Parkinson`s, Huntington`s and Alzheimer`s disease [25,39,42,96,65,69-72,85-87]. Furthermore, the pathogenesis of neurodegenerative diseases involve a gradual and progressive loss of neuronal cells [12,25,39,42,45, 65,69-72,85-87,96]. To a large extent, cell death leading to the nervous system dysfunction ensues by apoptosis but morphological, biochemical and molecular features of necrosis, autophagic cell death, among others, have been reported as well, apparently depending on the brain region/cell type affected and the type of primary malfunction/injury [12,25,39,42,45, 65,69-72,85-87,96]. The present review mainly addresses the question on the relation between initiation and execution of autophagocytosis in general and those molecular events that specifically might affect the life-death decision of cells.

Autophagy: a basic mechanism to maintain cell homeostasis

Tightly controlled degradation of surplus cellular components is essential as their biosynthesis for sustaining cell homeostasis. Cells own various catabolic pathways to cover a broad range of demands as elimination of small molecules up to complete organelles may become necessary, among which the ubiquitin-proteasome and the endosomal-lysosomal system are of particular interest in view of neurodegeneration (see below). The ubiquitin-proteasome system pathway plays an essential role in the controlled degradation of most short- and long-lived intracellular proteins in eukaryotic cells. Three enzymes, namely E1 ubiquitin-activating enzyme, E2 ubiquitin-carrier enzyme and E3 ubiquitin-protein ligase, act sequentially to conjugate ubiquitin to proteins, generally resulting in their degradation [3]. Autophagy is responsible for the elimination and recycling of bulk cytoplasmic constituent's incl. whole organelles. Based on the mechanism of autophagic vacuole formation and delivery of material to this compartment, three types of autophagy can be discriminated: **chaperone-mediated autophagy**, macro- and microautophagy. In chaperone-mediated autophagy, proteins containing a pentapeptide motif related to KFERQ are transported across the lysosomal membrane. The most prominent morphological manifestation of **macroautophagy** are double (or multiple)-membrane bounded vacuoles, the formation of which is highly conserved from yeast to humans [1,22,55,77,96]. Briefly, the macroautophagic pathway (for the sake of simplicity herein subsequently referred to as "autophagy") in mammalian cells starts with the sequestration of the cytoplasmic material to form an early autophagosome. Current concepts suggest that the membrane of the early autophagosomes derives from specialized membrane cisternae of not yet clarified origin, named "phagophore"; recruitment of membranes from the endoplasmic reticulum and trans Golgi network may contribute as well [22,32]. Autophagic vacuoles (autolysosomes) result from fusion of late autophagosomes with lysosomes; thereby, the final degradation of the sequestered cytoplasmic material is triggered. Cytoskeletal proteins are an integral part of this pathway; the sequestration requires intermediate filaments (cytokeratin and vimentin), the movement and fusion of lysosomes with the late autophagosomes requires the microtubular system [32]. All steps including the final degradation of the sequestered cytoplasmic material in autolysosomes are ATP-dependent [22,32]. **Microautophagy** means that the lysosome itself takes up cytosolic components (incl. macromolecules such as glycogen) and organelles by invagination; it appears not to be subjected to metabolic regulation [108].

Typically, cells exhibit a low basal rate of autophagy to maintain homeostasis (kinetic aspects are reviewed in 22]. Autophagy can be upregulated, for instance, to replenish amino acids and glucose pools for protein synthesis in response to

nutrient/growth factor deprivation, or to reorganize the cellular architecture during development, or to remove protein aggregates and other cytoplasmic constituents damaged by toxic injury [22,25,31,55,77].

In recent years, a tremendous progress has been achieved in elucidating the underlying molecular/biochemical events. The reader is referred to recent reviews addressing the transcriptional and translational control of autophagy [1,22,55,62,77,96]. Briefly, the mammalian target of rapamycin (mTOR) kinase is a major integration site for nutrient responses in eukaryotic cells. For instance, upstream of mTOR class I PI3K/Akt signalling molecules link receptor tyrosine kinases to mTOR activation, thereby inhibiting autophagy in response to insulin-like and other growth factor signals. A class III PI3K complex including beclin1/Atg6 controls autophagosome formation. Autophagy is also subjected to the regulation by heterotrimeric G proteins, other kinases and phosphatases as reviewed in detail elsewhere [1,22,96]. Downstream of mTOR kinase, more than a dozen gene products, referred to as ATG genes [49], have been found to tightly control initiation and execution of autophagy in yeast and eukaryotic cells [1,22,55,77,96].

Neurodegeneration: impairment of the ubiquitin-proteasome and endosomal-lysosomal system

Hallmarks of neurodegenerative diseases, including Amyotrophic Lateral Sclerosis, Alzheimer`s-, Parkinson`s- and Huntington`s disease or transmissible spongiform encephalopathies (prion diseases), are proteins that misfold and aggregate; impairment of the ubiquitin-proteasome as well as of the endosomal-lysosomal system is causatively involved [3,5,25,39,42,57,75,80,85-87,96]. In general, it appears that the ubiquitin-proteasome system plays a major role in reducing the levels of soluble misfolded proteins, while autophagy in clearing of cells from protein aggregates [25,75,96]. For instance, the early onset of Parkinson's disease involves inclusions (Lewy bodies) containing mutated alpha-synuclein as a major protein. In the cell culture, the expression of mutant but not wild-type alpha-synuclein was found to cause accumulation of autophagic vacuoles, along with impairment of the ubiquitin-proteasome system [101]. Notably, in brains of Parkinson's disease patients mutations in the ubiquitination system were also found; it may well be that the occurrence of ubiquitylated proteins as well as of components the ubiquitin-proteasome system itself in the inclusions result from unsuccessful attempts to remove aggregating proteins [3]. Likewise, studies with neural SH-SY5Y cells revealed that chronic low-level proteasome inhibition (by proteasome inhibitor MG115; 100 nM) may cause excessive activation of the endosomal-lysosomal system [3, 29]. On the other hand, Huntington's disease appears not to involve defects in the ubiquitin-proteasome system as eukaryotic proteasomes cannot digest polyglutamine sequences [107]. Rather, autophagy appears to be a major defence pathway as a block of mTOR and consequently, induction of autophagy attenuated accumulation of polyglutamines and cell death in experimental models of Huntington disease; inhibition of autophagy exhibited the opposite effects [86,107]. Furthermore, the brains of Alzheimer's disease patients revealed a massive induction of the endosomal-lysosomal compartment, as shown by a quantitative immunocytochemical-morphometric study [17]. In general, malfunction of the endosomal-lysosomal system is a prominent phenotype of inherited neurodegenerative disorders [17,87].

In summary, the pathogenesis of neurodegenerative disorders can be traced back, in part at the molecular level (such as specific mutations), to disturbance of the ubiquitin-proteasome and endosomal-lysosomal system. A controversial issue is whether the occurrence of intracellular aggregates results in cell death or, conversely, whether they are non-toxic and their presence reflects a cellular protective mechanism. In case of a causal relationship to neuronal cell death, is not clear how aggregated proteins such as alpha-synuclein, amyloid beta-protein, and huntingtin or their elimination by autophagocytosis eventually may be linked to cell death. In many neuronal pathologies apoptosis appears the predominant form of cell death, the participation of autophagy in cell death is poorly understood.

Concepts on cell death

The occurrence of cell death under a variety of physiological and pathological conditions in multicellular organisms has been documented many times during the past 160 years [20]. In pathology

and toxicology, based upon the pioneering work of Rudolf Virchow in the 1850s cell death (usually called "necrosis") in living organisms traditionally was considered a passive, degenerative phenomenon resulting from external insults by numerous agents. This view of cell death was revolutionized in the early 1970s by a group of British-Australian pathologists (John Kerr, Andrew Wyllie, Alistair Currie) proposing two broad cell death categories: 1. necrosis, which was re-defined and restricted to events caused by violent environmental perturbation leading to collapse of internal homeostasis. 2. The new term „apoptosis" (now often and in a broader sense called programmed cell death) was coined to describe an orchestrated collapse of a cell, staging membrane blebbing, cell shrinkage, chromatin condensation, DNA and protein degradation, accomplished by phagocytosis of corpses by the neighbouring cells [46,111].

Apoptosis gained considerable credit when it became clear that it constitutes an essential part of life for any multicellular organism and modern techniques provided insights into its molecular pathways; these revealed to be conserved from worm to mammal. Thus, in a number of biological settings apoptosis involves the action of caspases as major players. For instance, the typical morphology of apoptosis largely is the end result of caspase-mediated destruction of the cellular architecture [52,60,61]. Caspases belong to a large family of highly conserved proteins that have been found in hydra, insects, nematodes and mammals; a number of them constitute a set of sequentially acting "initiator" and "executioner" caspases mediating a wide range of physiological and non-physiological pro-apoptotic signals down to a final coordinated self-destruction of the cell. Mitochondria constitute a major site for integration of diverse pro-apoptotic signals ["intrinsic pathway" via caspase-9 activation (apoptosome) as opposed to "extrinsic pathway", triggered by activation of caspase-8 via death receptors of the TNF/NGF-family; both pathways join at the level of caspase-3; 52,53], but the endoplasmic reticulum [84], lysosomes [102] and the trans-Golgi-Network [64] play important roles as well. Thus, each organelle possesses sensors that detect specific alterations; locally activates signal transduction pathways and emits signals that ensure inter-organellar cross-talk.

Along with this gain in knowledge, however, morphological, biochemical and molecular observations revealed that active self-destruction of cells is not confined to apoptosis but cells may use different pathways to commit suicide, thereby severely challenging the initial necrosis-apoptosis dichotomy [13-15,21,31,48,56,60,61,96,100,104,110,113,116]. For instance, cell death induced by apoptotic stimuli such as CD95-L or TNF exhibit hallmarks of necrosis under conditions of caspase-inhibition ("programmed necrosis"; 31,113]. Moreover, caspase-independent cell death may also ensue with the morphology of apoptosis [60,61]. Notably, early morphological and histochemical studies revealed no evidence for autophagic or lysosomal events in apoptotic cells in vivo [16,46,111]. To date, the autophagic-lysosomal compartment has been implicated in the initiation of programmed cell death, either upstream or independent of caspase cascades, often denoted "type II programmed cell death" or "autophagic cell death" [6,13-15,21,37,60,61,91,116]. **Autophagic cell death**[1] is characterized by the degradation of cytoplasmic components incl. organelles preceding the nuclear collapse, but preservation of cytoskeletal elements until late stages [13-15; for early morphological description see 91]. Typical electron microscopical features of autophagic cell death as exemplified by human mammary carcinoma cells treated with the antiestrogen tamoxifen are depicted in figure 1. Briefly, the first changes visible at the electron microscope level comprise formation of autophagic vacuoles (AV), which gradually degrade cytoplasmic structures. In view of the attempt to elucidate biochemical/molecular specificities of the cells` various suicide pathways, the cytoskeleton deserves attention. Thus, depolymerization or caspase-driven cleavage of cytoskeletal proteins and their regulators are early events in apoptosis [52,53]. Cytoskeletal elements, however, are necessary for the autophagic process to ensue [32]. For instance, the sequestration of cytoplasmic structures involves the action of intermediate filaments. Accordingly, the histochemical and biochemical analysis of tamoxifen-induced autophagic PCD in MCF-7 cells revealed that the cytoskeleton was redistributed but largely preserved even in cells exhibiting nuclear condensation/fragmentation, i.e. the irreversible stage of cell death [13-15]. These observations with dying cells meet well with the current concept on macroautophagy (see above).

No evidence for the involvement of caspases was found in our studies and others [13-15,60,61].

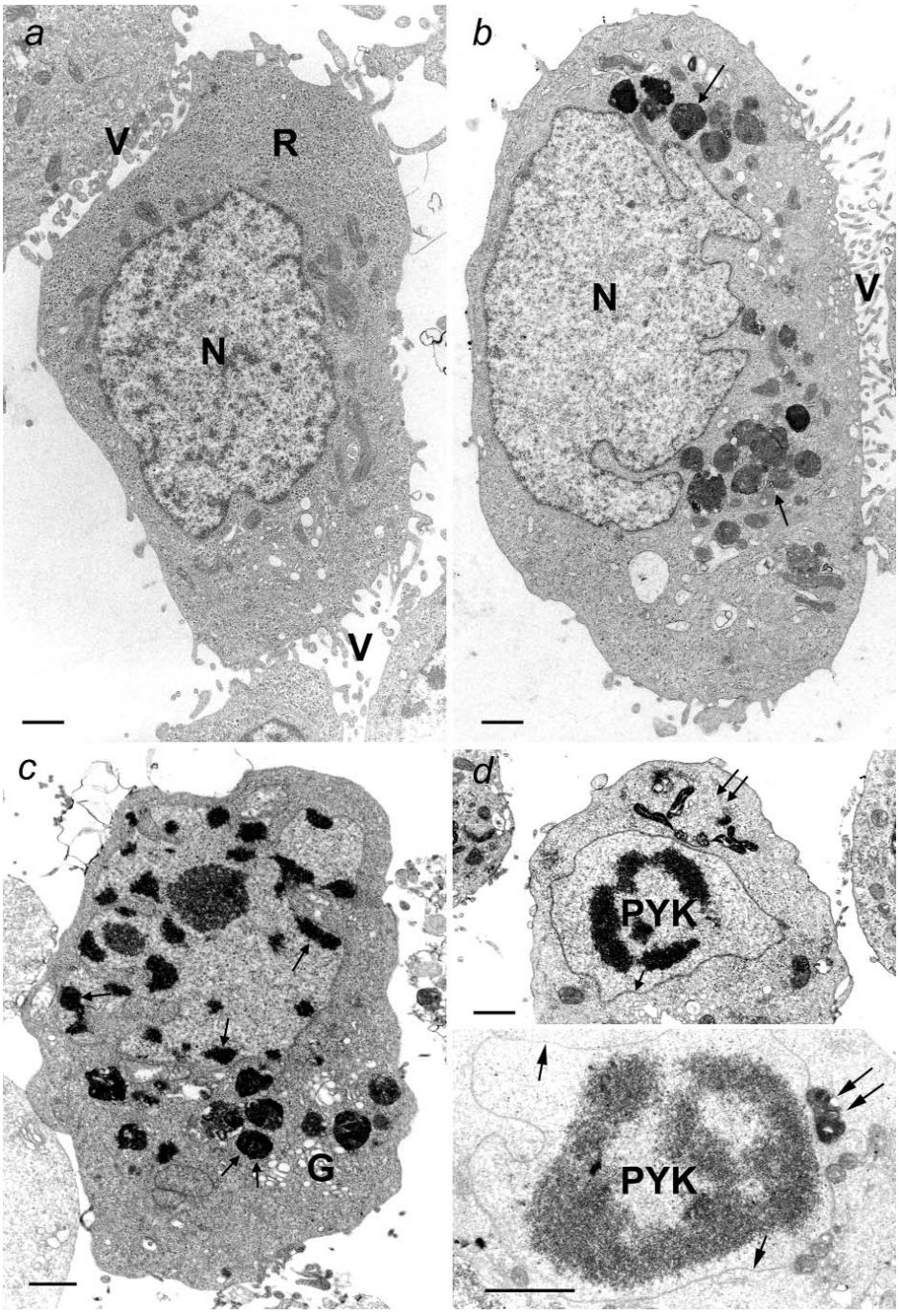

Fig. 1. Ultrastructural features of autophagic cell death in cultured human mammary carcinoma cells (MCF-7). (a) Control, day 7; intact nucleus (N), the plasma membrane exhibits extended areas with microvilli (V), the cytoplasm shows multiple polyribosomes (R). MCF- 7 cultures after TAM treatment (b-d). (b) 10^{-6} M TAM, day 7; the nucleus appears normal (N), in the cytoplasm numerous AVs (↑) are visible, microvilli are still present (V). (c) TAM 10^{-6} M, day 7; ribbons of condensed chromatin are detached from the nuclear envelope (↑); numerous AVs (↑↑) and prominent Golgi regions (G) are seen in the cytoplasm. (d) TAM 10^{-6} M, day 7; rounded cells, surface are characterized by loss of microvilli. The condensed chromatin is detached from the nuclear envelope and concentrated in the center of the nucleus (PYK); the nuclear envelope appears intact (↑). In the amorphous cytoplasm mitochondria and AVs are clustered at the cell poles/nuclear envelope (↑↑). Bars 1 μm

However, autophagic cell death does not always ensue in a caspase-independent fashion. For instance, cell death during *Drosophila* development includes autophagocytosis but also requires caspase activity [6,54,67]. Genetic screening of developmental stages of *Drosophila* revealed that elements of apoptosis and autophagy may be activated simultaneously; the affected genes comprise *ark* (Apaf-1 homologue), *dronc*, *drice* and *dream* (caspases), *buffy* (a bcl-2 family member), phagocytosis-related genes such as *crq* (croquemort) and the DNase *rep4* [6].

The occurrence of autophagic cell death cannot be assigned to specific biological conditions. Nevertheless, in adult organisms including humans, autophagy is often associated with death of (large secretory) cells during adjustment of sexual organs and ancillary tissues to seasonal reproduction. Furthermore, autophagy appears to be important in cell death under biological conditions of tissue remodelling such as insect metamorphosis; during vertebrate development, autophagic cell death is associated with organ morphogenesis as exemplified by shaping of extremities, cavity formation in the intestine and regression of the sexual anlagen. In all cases, the developmental program or (in adulthood) homeostatic mechanisms demand massive cell elimination. In instances of cell injury, damaged organelles or membranes may be transferred into the autophagic pathway, serving as a protective response at the subcellular scale and once a cell becomes overwhelmed, elimination of the whole cell may result [13,60].

In summary, it appears that diverse cell death programs emerged during evolution, the conservation of which apparently equips cells with a high degree of flexibility in assembling such elements to a cell death pathway according to the (patho)physiological conditions and needs.

Cell death in the neuronal system

Apoptosis of neuronal cells is essential for shaping and accurate wiring of the developing nervous system [8,69-72,113,114]. In general, developing neurons are committed to enter apoptosis unless they are rescued by neurotrophic factors [8]. Upon completion of development neurons need to survive and to reduce the probability of accidental cell loss, apoptotic pathways are downregulated and concomitantly, survival pathways may be upregulated [8]. For specific biological needs, however, programmed neuronal turnover may continue in adulthood as exemplified by neurogenesis of neuronal circuits involved in odor discrimination or in learning and memory. Like in regenerating skin and other organs, first an excess of neuronal progenitors is produced, followed by elimination of the vast majority of cells except those that differentiate into functional neurons [4,73]. As in many non-neuronal cell types, the principal molecular components of the apoptosis program in neurons include the two major pathways, namely the intrinsic and extrinsic pathway with their final outcome governed by the ratio of pro- and anti-apoptotic molecules [8, 52,53,69-72,114].

Defective apoptosis in the developing as well as adult nervous system, as caused by genetic or accidental factors, contributes to multiple neurodegenerative dysfunctions. For instance, ethanol has been found to trigger massive apoptotic neurodegeneration in the developing brain by interfering with both the NMDA and GABA receptor systems. These observations provide an explanation for the reduced brain mass and lifelong neurobehavioral disturbances associated with intrauterine exposure of the human foetus to ethanol (foetal alcohol syndrome; 78]. Other triggers for neuronal apoptosis comprise hyperactivation of glutamate receptors (excitotoxicity) in acute (e.g. stroke) and chronic disease such as Alzheimer's disease and motor system disorders [7,69,72].

Alzheimer's disease (AD) is characterized by gradual cell death in the hippocampus and the frontal cortex, eventually leading to severe memory loss. In Parkinson's disease, there is extensive loss of dopaminergic neurons in the substantia nigra, which results in a unique movement disorder. Amyotrophic lateral sclerosis is a condition in which motor neurons are selectively destroyed, leading to paralysis and eventual death. Huntington´s disease primarily involves the progressive death of GABA-ergic neurons of the striatum and in the deep layers of the cortex; during the later stages of the disease, the degeneration extends to a variety of brain regions, including the hypothalamus and hippocampus [12,45,69,72,74,113]. A large body of evidence suggests that apoptosis is a substantial contributor– although not the only one- to progression and pathology of these neurodege-nerative dysfunctions. For instance, progressive neurodegenerative changes

in the striatum, hippocampus and cerebellum of weaver mutant mice with typical Parkinsonism revealed features of apoptosis such as caspase-3 activation and inter-nucleosomal DNA fragmentation [30]. Likewise, in the pathogenesis of AD, both extracellular amyloid deposits and intracellular amyloid beta protein may activate caspases, leading to the cleavage of nuclear and cytoskeletal proteins, including the tau protein [24,28,80,103]; mutations in genes coding for presenilins control apoptotic signalling cascades and neuronal apoptosis [103]. As in many non-neuronal cells, endoplasmic reticulum stress also may play a central role in the execution of neuronal apoptosis in acute or chronic states of disease [80,94,103]. Finally, activation of p53 in response to the DNA damage, oxidative stress, metabolic compromise, cellular calcium overload can trigger apoptosis in neurons [26, 71].

Notably, acute and chronic stress imposed on mature neuronal cells, however, does not only result in activation of pro-apoptotic but also in the activation **survival** mechanisms, with NF-kappaB acting as a central player; this is considered a basic physiological means of fine-tuning to prevent too much cell loss by accidental apoptosis [71].

Pathological neuronal death is not confined to apoptosis, but morphological and molecular features indicate the involvement of necrosis, autophagic cell death as well as transitional phenotypes. **Necrosis** typically occurs following ischaemia, hypoxia, stroke or trauma but it has also been reported in Alzheimer`s`-, Huntington`s-, Parkinson`s-disease and Amyotrophic lateral sclerosis [5]. The development of necrosis involves an increase in intracellular calcium with the concomitant activation of cysteine calcium-dependent proteases (calpains); caspases appear not (always?) to be activated in necrotic cells as indicated by their resistance to caspase inhibitors [5].

Last, but not least, autophagy has been implicated in neuronal cell death. Reports on the occurrence of **autophagic cell death** in the nerve systems as well as other biological settings were summarized some years ago [13]. To update the list with a few examples, degenerating Purkinje cells in a lysosomal storage disease (Niemann-Pick type C, caused by mutations npc1 or npc2 gene) exhibit features of autophagic cell death [50]. Likewise, an ultrastructural study on neuronal degeneration in transgenic mice expressing mutant (P301L) human tau suggests the involvement autophagic processes [59]. Neurodegeneration in the lurcher mouse is caused by mutation of the GluRdelta2 gene that results in a constitutively active glutamate receptor ion channel. Neuronal death in this model was reported to be independent of depolarization and to be brought about by direct activation of autophagy by Lurcher GRID2 receptors through a signalling pathway formed by GRID2, n-PIST, and Beclin1 [93,115]. Autophagy has also been reported to be of importance in transmissible spongiform encephalopathies (TSEs) and may even participate in a formation of spongiform change [57]. A current matter of debate is how an anticipated anti-survival function of autophagy might meet with its well established protective function as exemplified by removing of misfolded, potentially dangerous proteins.

Does autophagy contribute to the life-death decision of cells?

The concept of autophagic cell death as a distinct entity is severely challenged by the fact that autophagocytosis constitutes a major inducible pathway for degradation of cytoplasmic components that in most cases result in survival rather than death of a cell. Therefore, morphological features as shown in fig. 1 are not sufficient to imply a causative relationship between autophagocytosis and eventual manifestation of a cell´s suicide. Consequently, a key question to be answered is whether autophagy might be just an epiphenomenon of cell death or whether specific functional links/molecular events enable autophagy to regulate distinct survival and death pathways.

Early studies aimed at elucidating a functional link between autophagocytosis and eventual cell death by inhibition experiments with 3-methyladenine (first described to inhibit sequestration of cytoplasmic components [92], wortmannin and LY294002 [33,81]. Briefly, these compounds have been found to prevent both, the formation of autophagic vacuoles and the eventual cell death (indicated by nuclear destruction) induced by cytokines, gene(over)expression, drugs, bacterial toxins in a variety of different cell types; [2,13,19,33,43,44,48,79,90,104]. Their inhibitory action on autophagocytosis involves class III PI3-Kinases (positive regulators of autophagy, catabolism) as well as class I PI3-kinases (negative regulators of autophagy) [2,33,81,104]. However, the action of 3-

MA is not limited to the sequestration step in autophagocytosis but also may affect apoptosis signaling [JNK and p38 kinases; mitochondrial permeability transition pore opening; 10,104]. Thus, according to the current state of knowledge, these inhibition studies are not sufficient to establish autophagocytosis as an integral part of a death pathway, but – taking into account recent findings (see below) -may be considered as supportive.

More recently, the first clues to uncover a crosstalk between signaling pathways for autophagy and cell death have been provided by studies on the autophagy genes Atg5, Atg7 and Beclin1 [56,83,115]. In HeLa and MCF-7 cells, Atg5 (which cooperates with LC3-II as major regulator of the sequestration step) was found to interact with FADD and thereby, to link autophagy to the execution of cell death [83]. Thus, ectopic expression of Atg5 induced autophagy preceding cell death; IFN-gamma treatment exhibited the same effects. Conversely, downregulation of Atg5 or expression of Atg5(K130R) mutant suppresses cell death and vacuole formation. Furthermore, both processes could be dissected as caspase-inhibition prevented cell death but not vacuole formation, i.e. at the apex of caspase cascades [83]. Most recently, Simon and coworkers [98] reported that in a cells overexpressing Atg5, a calpain cleavage product of Atg5 translocates to mitochondria and thereby, triggers cell death involving cytochrome c release and partial antagonism of Bcl-2 and Bcl-XL. Taken together, Atg5, a major regulator of the sequestration step in autophagy, may turn into a pro-apoptotic signal at two levels, 1st via death receptor adaptor molecule FADD (extrinsic pathway) and 2nd, via translocation of a calpain-cleavage product of Atg5 to mitochondria (intrinsic pathway). It is tempting to speculate that, if induction of autophagy primarily reflects a protective mechanism, this might be bypassed by truncated Atg5 in an elaborate way favouring the release of pro-apopotic and concomitantly, by antagonzing pro-survival factors. Other autophagy genes found to be required for cell death are Atg7 and Beclin1, as caspase-inhibitor induced cell death is attenuated by RNAi against these gene products [112]. Likewise, RNAi against Beclin1 and Atg5 prevented cell death of Bax/Bak double knockout cells treated with the apoptogenic compounds staurosporine and etoposide [95]. In summary, these examples show that in principle, induction of autophagy (including its transcriptional level), may be linked to the execution of cell death by making use of the apoptotic machinery. This conclusion is in line with other observations showing that both, the autophagic as well as mitochondrial compartment may be targeted by the same intrinsic pro-apoptotic signal as exemplified by DAP kinase [23,37]. Likewise, an immunocytochemical study on brain sections from AD patients suggested that apoptosis, along with autophagy, is subjected to the regulation of PKR-eIF2alpha [18]. Further examples comprise the anti-glioma action of the herbal anthraquinone derivative emodin, which was found to involve ERK-independent induction of both, apoptosis and autophagy [76]. Nutrient deprived LAMP2-negative cells exhibited an accumulation of autophagic vacuoles, followed by cell death with hallmarks of apoptosis (loss of the mitochondrial transmembrane potential, caspase activation, chromatin condensation; 36]. Histone deacetylase inhibitors, such as suberoylanilide hydrox amic acid, can trigger both mitochondria-mediated apoptosis and caspase-independent autophagic cell death [66].

However, the biology of cell death is much more complex as contrary observations have been made: inhibition of autophagy also may trigger apoptosis. For instance, Boya et al. [11] reported that genetic (by a small interfering RNA targeting Atg5, Atg6/Beclin1-1, Atg10, or Atg12) or pharmacological (by 3-methyladenine, hydroxychloroquine, bafilomycin A1, or monensin) inhibition of autophagy triggered apoptosis in the mammalian cell. Likewise, Bafilomycin A1, a specific inhibitor of vacuolar type H(+)-ATPase, blocked autophagy by inhibiting fusion between autophagosomes and lysosomes but triggered apoptosis through activation of caspase-3 with mitochondrial and lysosomal membrane permeabilization [44].

There is also evidence that in case of blocked apoptosis, cells may enter or complete their suicide via non-apoptotic mechanisms incl. autophagic pathway, conceivably serving as a back up mechanism. For instance, in neuronal cell death caspases are activated in most situations but in case of caspase inhibition at, or downstream of the apoptosome, neurons undergo a delayed, caspase-independent death [100]. Studies on Huntington´s disease gave rise to the hypothesis that prothymosin-α may provide a switch between

apoptosis and autophagy by a negative regulation of the apoptosome activity [82]. The idea of co-existence of autophagic and apoptotic death signaling in cells is supported by a few cases in which cells were found to switch between apoptotic and autophagic cell death pathways; as the manifestation of either phenotype depended on the extrinsic death stimulus, the decision appears to include the level of receptors. RAS-induced neuronal cell death exhibited elements of autophagocytosis, but apoptosis upon TNF-α [47,48]. Likewise in human breast carcinoma cells, the antiestrogen tamoxifen induced an autophagic death pathway [15]. However, upon TNF-α exposure these cells enter a death pathway involving the initiator caspase–8 at the apex of a caspase cascade including cleavage of cytoskeletal proteins [41,63]. Notably, as MCF-7 cells lack functional caspase-3, these findings demonstrate that the manifestation of either pathway is not just merely a consequence of lacking caspase 3. Nevertheless, these and other observations on caspase-dependent/independent cell death [reviewed in 15,61] gave a reason to ask whether the activity of caspases may be decisive for the manifestation of cell death phenotypes. In fact, the phenomena of "caspase-dependent/independent" forms of cell suicide initiated a controversial discussion, thereby revealing some important aspects of cell death biology:

1. Morphology of cell death

Caspases appear to constitute a major but not the sole determinant for the manifestation of the apoptotic morphology. Cell shrinkage and chromatin condensation characteristic of apoptosis (note: not (oligo)nucleosomal DNA-fragmentation) may ensue in a caspase-independent fashion. Alternative non-caspase proteases, acting either upstream or downstream of mitochondria comprise perforin/granzymes [105]; lysosomal cathepsins [40]; granzyme A [58]; calpains [34,84]. Mitochondria may release pro-apoptotic but caspase-independent effectors such as apoptosis inducing factor (AIF), endonuclease G, as well as Omi/HtrA2, which possess serine protease activity [60,61,68].

The morphology of caspase-independent cell death may range from "apoptosis-like" to "necrosis-like", as denoted by Mathiasen and Jäättelä [68]. Thus, the envision of caspase-independent PCD pathways may help to integrate seemingly conflicting observations/interpretations of cell death morphologies into experimentally testable biochemical/molecular concepts on cell death biology, incl. autophagic cell death.

2. Initiation and execution of cell death

Caspase-dependent and caspase-independent pathways may co-exist in the same cell and even may be co-activated [60,61]. A protein may possess a bi-functional role by being involved in caspase-dependent as well as caspase-independent cascades. For instance, mature Omi can induce apoptosis in human cells in a caspase-independent manner through its protease activity and in a caspase-dependent manner via its ability to disrupt caspase-IAP interaction [38].

3. Autophagy

In a number of cases with mammalian cells the autophagic type of cell death ensues independent of caspases [15,60,61]. Furthermore, vacuolar (autophagic) programmed cell death in *Dictyostelium* neither requires meta- nor paracaspases [89]. However, in insects cell death associated with autophagy was found to involve caspases. For instance, in Manduca sexta accessory planta retractor motoneurons undergo dendritic loss at the end of larval life; cell death occurs by autophagy, not apoptosis, involving caspase activation and the aggregation of mitochondria [109]. Likewise, salivary gland cell death during Drosophila development includes autophagocytosis but also requires caspase activity [see above; 6,54,67]. Thus, the criterion of functional/non-functional caspases cannot be used to distinguish autophagic cell death from other cell death mechanisms.

Conclusions

Multiple, evolutionary conserved suicide pathways are available in higher eukaryotic cells; ancient molecular cell death mechanisms have been improved by acquiring complex sets of interacting "death" and "survival" molecules that allow a higher eukaryotic cell to finely tune its life-death decision [51,110]. For instance, recent cDNA microarray analysis of over 1000 brain-related genes revealed a complex pattern of activation and inactivation of seemingly unrelated genes responsible for regulation of neuronal excitability, inflammation, cell death pathways, cell adhesion and transcriptional activation [7].

Programmed cell death can be activated by a broad spectrum of well-known extrinsic and intrinsic signals; the molecular regulatory network,

however, leaves many questions open. From a teleological point of view multiple suicide pathways such as caspase-dependent and caspase-independent apoptosis or autophagic cell death seem to be of advantage for the organism: a cell would be equipped with distinct, but interchangeable sets of enzymes to commit suicide. Alternative pathways to caspase-dependent apoptosis include condensation of dying cells as it may facilitate their phagocytosis. It is tempting to speculate that autophagic cell death might reflect a complementary strategy to protease-driven cell death, not relying on precise cleavage of a limited set of crucial proteins but relying on removal of bulk cytoplasmic constituents prior to final removal through phagocytosis. Thus, the phenotypic variations of programmed cell death reflect the organism's flexibility to respond to physiological and non-physiological demands.

As to autophagy, sublethal damage clearly activates autophagic defence; in case this protective mechanism is overwhelmed or cannot be completed properly, elimination of the whole cell might ensue. Most recently it was found that, in principle, induction of autophagy genes may be linked to execution of cell death (or render cells more susceptible to death signals) by making use of elements of the extrinsic or intrinsic apoptosis pathway. These observations showed that molecules -most likely predominantly- steering autophagy may exert distinct function(s) in cell death pathways. However, the cascade from autophagy to apoptosis is not a one-way street as the converse may occur.

Taken together, there is a considerable overlap between cell death signaling pathways and consequently, a clear-cut distinction between apoptosis and autophagic cell death at the biochemical/molecular level is difficult or even impossible; with respect to cell death nomenclature probably no consensus, except some general criteria, will be achieved. It should be reminded that the term "apoptosis" originally was coined on morphological grounds (cf. footnote 1). However, the morphological visible stages cell death (electronmicroscopy, demonstration of autophagic vacuoles by histochemical means, among others) in general correspond to relative late events, i.e. the outcome of the complex death signaling. Apparently, the final destruction of the cellular architecture ("final common pathway") results in phenotypes of some less diversity, which should not be mixed up with the complexity of the upstream molecular regulatory network. Nevertheless, irrespective of unresolved nomenclature, dissecting distinct elements of cell death signaling and their crosstalk is *conditio sine qua non* to refine therapeutic strategies based upon modulation of cell death, in case of neurodegenerative disorders to favour survival. Thus, successful and promising neuroprotection by interference with neuronal apoptosis has been observed in a number of experimental and clinical settings [9,27,72,97]; elucidation of new target molecules favouring cell death upstream or complementary to "classical" apoptotic pathways give hope to achieve additive or even synergistic effects. As to the role of autophagy in cell death, tools that became available in recent years, such as yeast autophagy genes along with their orthologs in mammals, may open new experimental avenues to tackle pertinent hypotheses.

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Because of the rapid progress in cell death research with its large number of publications it is not possible to cover all in detail. Therefore, we referred to review articles whenever possible and apologize to the many authors whose original publications are not cited directly.

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[1] In the present paper electron microscopical demonstration of autophagic vacuoles (AVs) in dying cells is taken as conditio sine qua non to denote cell death as autophagic cell death. In addition, histo- and biochemical criteria indicating a role of the autophagosomal-lysosomal compartment can be taken into account as reviewed in detail elsewhere [13]. It should be emphasized that referring to the morphological/histochemical features does not imply a causative relationship between autophagocytosis and eventual manifestation of a cell´s suicide; this will require either an established functional link between these phenomena and/or elucidation of specifically related genetic/epigenetic events. The term apoptosis originally was coined on morphological grounds (notably, excluding self-digestion of the affected cell [46,111]). Therefore, for the time being we apply the same category of criteria as the discriminator for autophagic cell death.