Cell Death and Differentiation (2001) 8, 569–581
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Review

The autophagosomal–lysosomal compartment in programmed cell death

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Received 3.10.00; revised 19.1.01; accepted 1.2.01
Edited by M Piacentini

Abstract

In the last decade a tremendous progress has been achieved in understanding the control of apoptosis by survival and death factors as well as the molecular mechanisms of preparation and execution of the cell's suicide. However, accumulating evidence suggests that programmed cell death (PCD) is not confined to apoptosis but that cells use different pathways for active self-destruction as reflected by different morphology: condensation prominent, type I or apoptosis; autophagy prominent, type II; etc. Autophagic PCD appears to be a phylogenetically old phenomenon, it may occur in physiological and disease states. Recently, distinct biochemical and molecular features have been be assigned to this type of PCD. However, autophagic and apoptotic PCD should not be considered as mutually exclusive phenomena. Rather, they appear to reflect a high degree of flexibility in a cell's response to changes of environmental conditions, both physiological or pathological. Furthermore, recent data suggest that diverse or relatively unspecific signals such as photodamage or lysosomotropic agents may be mediatd by lysosomal cysteine proteases (cathepsins) to caspases and thus, apoptosis. The present paper reviews morphological, functional and biochemical/molecular data suggesting the participation of the autophagosomal–lysosomal compartment in programmed cell death. *Cell Death and Differentiation* (2001) **8,** 569–581.

Keywords: autophagic cell death; autophagic vacuoles; BID; caspase-independent PCD; cytoplasmic vacuolisation; endoplasmic reticulum

Abbreviations: Apg-genes, autophagy-defective genes; ASP, apoptosis specific protein; PCD, programmed cell death; PI3-K, phosphatidylinositol-3-kinase; MSDH, O-methyl-serine dodecylamide hydrochloride; RID, receptor internalization and degradation; ROS, reactive oxygen species; TAM, tamoxifen; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α; TOR, target of rapamycin; 3-MA, 3-methyladenine

Diversity of programmed cell death: morphological evidence of autophagic cell death in states of health and disease

In the last decade apoptosis attracted growing interest of the scientific community and a tremendous gain in knowledge concerning the molecular events of its signalling, preparation and execution has been achieved (for review:[1–6]). However, accumulating morphological and biochemical evidence suggests that programmed cell death (PCD) is not confined to apoptosis but that cells use different pathways for active self-destruction: condensation prominent, type I or apoptosis; autophagy prominent, type II PCD etc.[1,7–12] In particular, according to the original morphological and histochemical based description of apoptosis, the autophagosomal–lysosomal system was considered not to play a role in initial stages of apoptosis.[13,14] Rather, the action of lysosomes appeared to be restricted to the (heterophagic) degradation of apoptotic bodies ensuing after phagocytosis by vital cells.[13,14] Thus, phagocytosis of apoptotic cell residues constitutes an integral part of the overall suicide process,[5] the molecular aspects of which are reviewed elsewhere in this issue of Cell Death and Differentiation. In the present paper, first morphological, functional and molecular features of autophagic cell death (type II PCD) will be reviewed. Secondly, recent evidence indicating an important function of lysosomal cysteine proteases in the preparatory stages of apoptosis (type I PCD) such as processing of BID and caspases will be addressed.

Reviewing the literature revealed a non-consistent use of terms to describe cell death associated with autophagocytosis as it includes necrosis, non-apoptotic type of cell death, apoptosis/type I PCD, autophagic cell death/type II PCD and others (Table 1).[15–38] For the purpose of summarising the morphological evidence of its occurrence in states of health and disease, electronmicroscopical demonstration of autophagic vacuoles in dying cells was taken as *conditio sine qua non* to denote cell death as autophagic/type II PCD; in addition, available histo- and biochemical criteria indicating a role of the autophagosomal–lysosomal compartment were included into Table 1. It should be emphasised, that referring to the morphological/histochemical features does not imply a causative relationship between macroautophagocytosis and eventual manifestation of a cell's suicide; data suggesting a functional link between these phenomena as well as related molecular events will be discussed in subsequent paragraphs.

Autophagic cell death appears to be a phylogenetically old phenomenon as it has been observed in the slime mold *Dictyostelium discoideum* and in the nematode *C elegans* (Table 1);[15–17] it even might have developed before

Table 1 Occurrence of autophagic cell death – *in vivo* (examples)

Species	(Patho)physiological condition and affected cell type	Features of cell death: Autophagosomal–lysosomal compartment	Features of cell death: Other	Description by authors
Fungi imperfecti *Dictyostelium discoideum*	Starvation: sorocarp formation (stalk cells)[15,16]	Cytoplasm: AV (EM)	Cytoplasm: condensation (EM); Nucleus: chromatin condensation (EM), no DNA fragmentation (BC); Caspase-independent (BC)	Programmed cell death
Nematodes *C. elegans*	Gain of function mutation: mec-4, deg-1 induced neuron degeneration[17]	Cell membrane: infoldings; Cytoplasm: formation of whorls and internal vacuoles, progressive loss of electron-density (EM)	Cytoplasm: swelling and vacuolization; Nucleus: condensation (subsequent to cytoplasmic alterations); Mitochondria, ER, Golgi: unaffected until late stages (EM); independent from ced-4, ced-3 (genetic analysis)	Necrosis-like cell death
Insects *Manduca sexta*	Metamorphosis: intersegmental muscle[18], labial gland[19]	Cytoplasm: AV (EM): Acid phosphatase activity: increase (BC, HC)	Mitochondria: persistence (early) of respiration (MTT-assay, ATP-content); SER: disappearance (EM) Nucleus: nucleosomal DNA fragmentation, ISEL positive	Autophagic degneration: type 2 PCD
	Prothoracic glands[20]	Cytoplasm: AV (EM);	Nucleus: condensation (EM), TUNEL positive Cytoplasm: disappearance of SER, fragmentation into membrane-bound bodies, their phagocytosis by hemocytes	Degenerating prothoracic gland cells display characteristics both, apoptosis and autophagy (confirmed by organ culture study)[21]
Calliphora vomitoria	Metamorphosis: salivary gland[22]	Cytoplasm: AV (EM) Acid phosphatase activity: increase (HC)	Cytoplasm: swelling Nucleus: no chromatin margination as in apoptosis; Mitochondria: persistence until fragmentation of cell	PCD distinct from classical apoptosis
Drosophila melanogaster	Metamorphosis: salivary gland[23]	Acid phosphatase activity: increase (HC) Ultrastructural distribution pattern: lysosomes, Golgi elements, multivesicular bodies, RER (within and on extracisternal surface)		Histolysis
Orgyia leucostigma	Adults: reduction of wing size (epithelial wing cells, femal, but not male)[24]	Cytoplasm: AV, lysosomes: increase (EM)		Programmed cell death
Birds	Development: limb bud morphogenesis in chicken regression of mesenchym[25]	Cytoplasm: AV: increase (EM); Acid phosphatase activity (HC);	Genetically controlled as indicated by mutants resulting in inhibition (poly-, syndacytly) or increase (reduced metacarpals, -tarsals) of PCD; Simultaneously: apoptosis (EM)	Necrosis (PNZ=posterior necrotic zones)
Rats, mice rabbits	Development: epithelial deterioration in uterus during implantation of egg, regression of interdigital webs, sexual anlagen, intestine (cavity formation)[7,8,25,26]	Cytoplasm: AV (EM) Acid phosphatase activity: increase (BC, HC)	For details: [5–7,9,17]	Autophagic degeneration, Type 1,2 necrosis, type 1,2 programmed cell death

(Continued)

Table 1 Occurrence of autophagic cell death – *in vivo* (examples)

Species	(Patho)physiological condition and affected cell type	Features of cell death: Autophagosomal–lysosomal compartment	Features of cell death: Other	Description by authors
Rats	Development: tooth eruption[27] (1) enamel epithelium, osteocytes, bone lining cells		(1) Cytoplasm: condensation, most organelles intact (EM), fragmentation into electron-dense apoptotoic bodies Nucleus: apoptotic-like condensation/fragmentation, TUNEL positive	(1) Apoptosis and (2) autophagic cell death (according to Clarke 1990).[7] In addition: dissolution of cytoplasm which was not considered in Clarke's classification, surmised to be one of the types of cell death in tooth development
	(2) resorbing bony crypt	(2) Cytoplasm: AV (EM)	(2) Cytoplasm: electron-lucent, fine granular structures, other organelles than autophagosomes seldom;	
Birds	Adults: follicular atresia, granulosa cells (Japanese quail)[28]	Cytoplasm: AV, formation simultaneously with nuclear alterations(EM)	Apoptosis (EM, Nuclei: ISEL positive) Detached cells, (cytoplasmic disintegration, small irregular clumps of chromatin indicative of primary necrosis (EM)	Three different types of cell death: – apoptosis – autophagic cell death – primary necrosis
Rat	Adults: mammary gland post weaning prostate post castration[11]	Cytoplasm: AV (EM)	Apoptosis (EM); Mixed type: apoptotic and autophagic features in same cell (EM)	Type 1,2, PCD, mixed type PCD
Rat	Adults: pulp of growing incisor with continuous cycle of growth, remodelling, regression and decay: Vascular smooth muscle cell involution of incisial arterioles[29]	Cytoplasm: AV (EM)	Autophagic PCD: shedding of cell fragments. Phagocytic vacuoles in neighbouring smooth muscle cells and adventitious macrophages (EM) Apoptosis: transformation of poly- into monoribosomes, nuclear and cytoplasmic condensation, fragmentation and phagocytosis (EM)	Apoptosis and autophagic type of cell death
Human	Adults: small intestine (duo-denun, jejunum): enterocytes at the villus tips[30]	Cytoplasm: AV, skrinkage (EM)	Nucleus: chromatin condensation immediately before exfoliation	Exfoliation
Human	Adults: endometrium: menstrual cycle day (27/28)[31]	Cytoplasm: AV (EM)	Golgi: decrease in size; Mitochondria: reduced number	Lamina functionalis in part regressed by focal death (autophagic vacuoles) or total death (apoptosis) of individual cells, both processes leading to phagocytosis of the cell debris by macrophages
Mice	Disease: weaver gene mutant[32] post-natal development External germinal layer of cerebellum		Apoptosis (EM) Nuclei: nucleosomal fragmentation, ISEL-positive, Increase in c-Jun, PCNA (all as in wild-type mice)	Apoptosis

(Continued)

Table 1 Occurrence of autophagic cell death – *in vivo* (examples)

Species	(Patho)physiological condition and affected cell type	Features of cell death: Autophagosomal–lysosomal compartment	Features of cell death: Other	Description by authors
Mice	Dopaminergic cells	Cytoplasm: AV (EM)	Nucleus: neither chromatin condensation (EM) nor DNA fragmentation (ISEL-negative) No increase in c-jun, PCNA	Combination of vaculoar and autophagic changes identifies a novel non-necrotic, non-apoptotic death
Human	Disease: Alzheimer[33,34]	Cytoplasm: AV (EM); Lysosomal enzymes: increase (HC)	Cytoplasm: accumulation of tertiary residual bodies (lysosomes, lipofuscin), (HC); Extracellular space: lipofuscin aggregates, associated with β-amyloid protein (EM, HC)	Chromatolysis, cell lysis
Human	Disease: Parkinson[35] progressive loss of dopaminergic neurons of the substantia nigra	Cytoplasm: AV (EM) Nucleus: chromatin condensation less pronounced as in apoptosis	Mitochondria: intact; Simultaneously: Apoptosis (EM)	Both, apoptosis and autophagic degeneration in melanised neurons of substantia nigra.
Human	Disease: cardiocyte loss during chronic haemodynamic overload[36]	Cytoplasm: secondary lysosomes in hyper- and atrophic cardiocytes (EM, HC)	Nucleus: DNA fragmentation (ISEL-positive)	Chronic self-controlled cytoplasmic proteolysis in cardiocytes, not initially associated with either nuclear degradation or intercellular dehiscence but later possibly accompanied by apoptotic nuclear elimination, and leading to apoptotic cell death
Rat	Toxic injury: CA1 pyramidal neurons in the hippocampus, ischaemia[37]	Cytoplasm: increase in volume density of cathepsin B-positive lysosomes and AVs (EM, HC)	Nuclei: strand breaks (ISEL-positive), oligo-nucleosomal DNA fragmentation, dense chromatin masses (EM)	The results suggest that delayed death of the CA1 pyramidal neurons after brief ischaemia is not necrotic but apoptotic.
Rat	Pancreatic acinar cells, vinblastine (10 mg/kg)[38]	Cytoplasm: AV (EM)	Nucleus: apoptotic-like condensation (EM)	Protein synthesis and enhanced autophagy may be indispensable step(s) in apoptosis in this system

AV: autophagic vacuole; SER/RER: smooth/rough endoplasmic reticulum; PCD: programmed cell death; EM: electron microscopy; LM: light microscopy; TUNEL, ISEL: *in situ* end labelling techniques for detection of single and double strand breaks in DNA. BC: biochemistry, analysis of tissue homogenate; HC: histo- incl. immunocytochemistry, analysis of individual cell using specific subtrates, antibodies etc. which allows discrimination between vital, dying cells, cell type affected; BC, HC: for the sake of shortness methods (endpoints) are not specified, see references for details. Description by authors: conclusions of authors word-for-word except abridgements

apoptosis.[10,39] A large body of morphological, histo- and biochemical evidence for autophagic cell death *in vivo* was provided by developmental biology. Insect metamorphosis may be considered as one of the most extreme biological conditions of tissue remodelling including autophagic cell death; cells of ecto-, endo and mesodermal origin are affected.[7,9,11,18–23] Likewise, in vertebrate development, autophagic cell death appears to be a prominent feature associated with organ morphogenesis as exemplified by shaping of extremities, cavity formation in intestine and regression of sexual anlagen.[7,8,11,25] Autophagic cell death also has been reported to occur in adulthood of insects and vertebrates including humans; it is often associated with the elimination of (large secretory) cells during adjustment of sexual organs and ancillary tissues to seasonal reproduction.[7,8,11,26,28,31] As to pathophysiology, autophagic cell death has been associated with experimental and human neurological diseases[32–35] as well as cytotoxic drug treatment.[38]

In summary, without attempting to force the data, the morphological observations strongly support the concept that autophagic cell death is characterised by features different from apoptosis (Figure 1) and which is a phenomenon of general importance occurring in a broad spectrum of (patho)physiological conditions. The most prominent features of autophagic cell death comprise degradation of cytoplasmic components resulting in progressive loss of electrondensity; the descriptions of autophagic cell death consistently include that degradation of cytoplasmic components precede nuclear collapse. Notably, the number of mitochondria in the cytoplasm decreased but those present in cytoplasm appeared intact; conceivably, the remaining mitochondria maintain ATP-levels required for the completion of autodigestion (see below and Figure 2). Like apoptosis, autophagic cell death has been described to be completed by phagocytosis.[7–9,18,20,29,31] In line with the general function of macroautophagy, namely being the major inducible pathway for degradation of cytoplasmic components including whole organelles, autophagic PCD predominantly appears to be activated when the developmental programme or in adulthood, homeostatic mechanisms demand massive cell elimination; in all cases, the bulk of cytoplasm is removed by autophagy before nuclear collapse ensues. In instances of cell injury, damaged organelles or membranes may be transferred into the autophagic pathway, serving as homeostatic mechanism at the subcellular scale, and that might be overwhelmed resulting in elimination of the whole cell. It is tempting to speculate that autophagic elimination of potentially harmful subcellular structures might be a functional analogue to the cell's safeguards controlling DNA repair and p53-mediated apoptotic suicide upon DNA damage. Finally, it should be noted that autophagic cell death and apoptosis are not mutually exclusive phenomena. Thus, both types of cell death can occur simultaneously in tissues,[7,11,27–29,35,38] but also subsequently as governed by the developmental programme.[31] Moreover, dying cells may share apoptotic and autophagic features ('mixed type').[7,11,20,21,37,38,40–44] The determinants for the eventual manifestation of either type of programmed cell death are poorly understood.

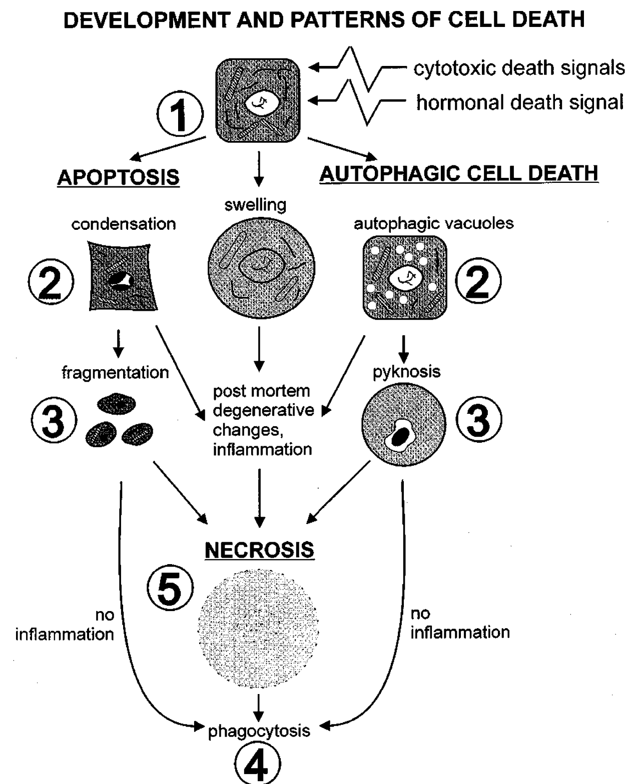

Figure 1 Development and patterns of cell death. (1) Commonalities of apoptosis and autophagic cell death: see text. Apoptosis: (2) condensation of cytoplasm and of chromatin at the nuclear membrane to sharply delineated masses (often like crescents). (3) cell fragmentation into apoptotic bodies. (4) Phagocytosis (*in vivo*) and heterophagic degradation. Note: according to original description autophagy/lysosomes do not play a distinct role early in apoptosis. Autophagic cell death: (2) Autophagy: formation of autophagic vacuoles (AVs; open circles) and degradation of cytoplasmic constituents; (3) Pyknosis, single pyknotic mass in the centre of the nucleus, nuclear envelope still intact, cytoplasm amorphous with few clusters of AVs and mitochondria (as observed in MCF-7 cells). Note: autophagocytosis with apoptotic-like DNA condensation/fragmentation may also occur; (4) Phagocytosis (*in vivo*) and final degradation. (5) A cell may enter apoptosis or autophagic cell death which, however, may not be completed and secondary necrosis ensues. For references: see text

Autophagic PCD: from morphology to molecular events

Current concepts on macroautophagy suggest that it ensues through a sequence of morphological visible events which are highly conserved from yeast to humans (for review:[39,45,46]). Briefly, the macroautophagic pathway in mammalian cells starts with the sequestration of cytoplasmic material to form an early autophagosome (Figure 2). The double-membrane of the early autophagosomes is generally considered to derive from ribosome-free regions of the endoplasmic reticulum; alternatively, it has been suggested to originate from a related organelle named 'phagophore' or from post-Golgi membranes (for review:[39,45,46]). Autophagic vacuoles (autolysosomes) result from fusion of late autophagosomes with lysosomes;

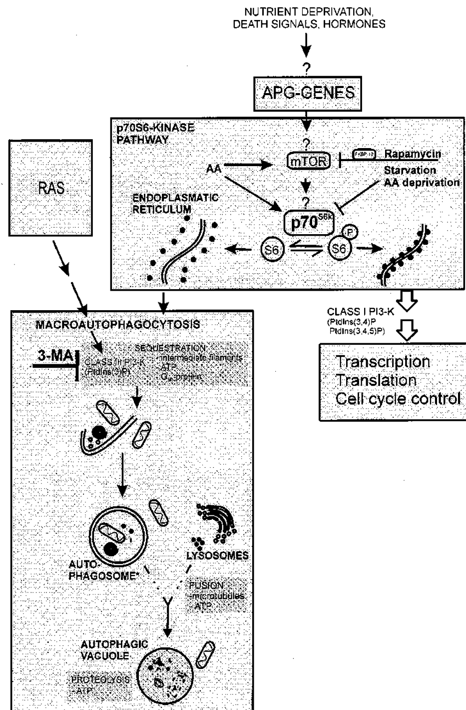

Figure 2 Macroautophagocytosis: concepts on regulation and morphological manifestation. Macroautophagy: For description see text. Note: simplified presentation as early and late autophagosomes, acidification, integration of autophagosomal membrane into that of autophagic vacuole etc. are not indicated (for review:[39,45,46]). Furthermore, cytoplasmic components may also be degraded by microautophagy, crinophagy, hsc73 chaperone-mediated autophagy, or non-lysosomal pathways including the ubiqutin-proteasome pathway and calpains as reviewed elsewhere.[46–48] p70S6kinase pathway: Schematic diagram following Dennis *et al.*,[61] (see text for explanation and references). APG genes: autophagic defective genes; mTOR: mammalian target of rapamycin. Rapamycin complexes with FKBP-12 (FK506 binding protein) to inhibit mTOR-phosphorylation. $p70^{S6k}$: p70S6-kinase; hypophosphorylated 70^{S6k} promotes detachment of ribosomes from RER and sequestration/autophagy; in contrast, hyperphosphorylated $p70^{S6k}$ promotes attachment of ribosomes to ER. →: Stimulation; ⊣ inhibition

thereby, the final degradation of the sequestered cytoplasmic material is triggered (Figure 2). 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 (for review[39,45,46]). All steps including the final degradation of sequestered cytoplasmic material in autolysosomes are ATP-dependent (Figure 2; for review:[39,45,46]).

A functional link between macroautophagoytosis and cell suicide could be established by inhibition experiments with 3-methyladenine (3-MA).[40,44,49–54] 3-MA has been described to specifically block the sequestration step;[49] at the molecular level 3-MA has been found to inhibit class III phosphatidylinositol-kinase activity (Figure 2).[54] Thus, 3-MA has been found to inhibit both, the formation of autophagic vacuoles and the eventual cell death as indicated by nuclear destruction in a number of experimental settings including tamoxifen treated human mammary carcinoma cells (MCF-7), gastric and glioma cells overexpressing Ras, TNF-α treated human T-lymphoblastic leukaemic cells, neuronal cells upon serum withdrawal or arabinoside, kidney cells lines treated with bacterial toxins such as ricin, abrin, Shiga toxin and diphtheria toxin.[40,44,49–53] In view of functional criteria for the differentiation between subtypes of PCD, it is of interest to mention preliminary data suggesting that 3-MA does not inhibit TGF-β1 induced apoptosis of hepatocytes (W Parzefall, personal communication).

Furthermore, possible interactions of mechanisms controlling the biogenesis of lysosomes[55] and their subsequent fusion with autophagosomes with those of the cell's suicide have to be considered. Early studies on regressing endocrine-dependent tumours suggested the involvement of *de-novo*-synthesis and an increased activity of lysosomal enzymes.[56] Both, the increment in number and activity of lysosomes were considered to be the effect, not the cause, of tumour regression.[56] More recently, TNF-α was found to induce an autophagic type of cell death in T-lymphoblastic leukaemic cells; 3-MA inhibited both the formation of autophagosomes and cell death.[50] However, asparagine, which inhibits the fusion of lysosomes with autophagosomes, did not prevent TNF-α induced cell death.[50] Thus, inhibition of an event downstream of sequestration (Figure 2) did not affect the execution of autophagic cell death, suggesting that the supply of lysosomes might not be a check point for initiation of this type of programmed cell death. Likewise, tamoxifen-induced autophagic cell death in MCF-7 cells was not associated with an expansion of the lysosomal compartment as indicated by biochemical and histochemical means (Török and Bursch, unpublished observation). Furthermore, 3-MA is known to slightly increase lysosomal pH in hepatocytes.[51] However, increase of the lysosomal pH by monensin or NH_4Cl did not protect kidney cells against ricin-induced lysis, thus excluding a possible increase in lysosomal pH as cause for the protective action of 3-MA.[51] A cautionary note on the role of lysosomal enzymes during cell death has been published recently.[57] Beem *et al.*[57] suggested that misconceptions may emerge because of preparatory artefacts resulting in breakage of apoptotic bodies during tissue homogenisation. It may well be that breakage of apoptotic bodies during homogenisation of tumours is the cause for an observed increase in soluble beta-glucuronidase activity, while the lysosomes of the ingesting tumour cells remain intact.[57] In summary, at present the interactions of lysosome biogenesis with the pathway(s) leading to autophagic cell death remain elusive. However, the current data based on functional criteria suggest that the sequestration step might provide a superior regulatory link between autophagocytosis and cell suicide rather than downstream events in the autophagic pathway.

As to biochemical characteristics, recent evidence suggests that the cytoskeleton exhibited distinct fates during autophagic and apoptotic cell death. In apoptosis, the cell's preparatory as well as executional steps include depolymerisation or cleavage of actin, cytokeratins, lamins

and other cytoskeletal proteins, most probably resulting in the typical final shape of apoptotic cells (details and references in[2,58]). In contrast, as exemplified by autophagic death of MCF-7 cells after tamoxifen, the cytoskeleton was found to be redistributed but largely preserved, even in cells exhibiting nuclear condensation/fragmentation (i.e. irreversible stage of cell death).[58] A pronounced fragmentation of the cytokeratin could not be detected before MCF-7 cells detached from the substrate and therefore probably were in a stage of secondary necrosis (Figure 1).[58] Remarkably, the vast majority (about 85%) of MCF-7 cells exhibiting a pyknotic nucleus still contained F-actin as demonstrated by its interaction with phalloidin.[58] Polymerisation of G- to F-actin is an ATP-dependent process and therefore, F-actin is a sensitive indicator of the metabolic state of a cell. In support of this notion, electronmicroscopy and rhodamine 123 staining revealed that at late stages of the death process the cytoplasm appeared amorphous, but the few remaining autophagic vacuoles were associated with clusters of structurally and functionally intact mitochondria.[44,58] The preservation of mitochondria and thus ATP synthesis throughout autophagic degradation was also supported by the observation that mitochondrial dehydrogenase-activity did not decrease with time in TAM treated MCF-7 cultures (Török and Bursch, unpublished observation). It appears likely, that ATP synthesis is maintained at a level required for the completion of autophagocytosis. Moreover, the protein cross-linking enzyme transglutaminase, which is activated in apoptotic hepatocytes,[59] is not involved in tamoxifen induced PCD of MCF-7 cells. Thus, the preservation of the cytoskeleton during autophagic death of MCF-7 cells matches with current concepts on the cytoskeleton's function in macroautophagy and furthermore, strongly supports the morphological evidence that apoptosis and autophagic reflect distinct pathways of cell suicide.

The genetics and signalling of macroautophagy is best studied in yeast, but – like its morphological appearance – the molecular events of initiation and execution of macroautophagy have been found to be highly conserved from this organism to humans.[39,46,60,61] Following Dennis *et al*.[61] a hypothetical model on molecular control of autophagy based upon yeast and mammalian data is depicted in Figure 2 ('p70S6-kinase pathway'). In yeast, to date 14 Apg-genes (autophagy-defective genes) are known to act in a conjugation cascade as reviewed elsewhere.[60] Two mammalian homologues of the Apg gene family have been identified, namely ASP/hApg5 (ASP: apoptosis specific protein;[62] and Apg6/vps30 (beclin-1).[63,64] ASP/hApg5 was first described as 'apoptosis specific protein' because of its expression in Burkitt's lymphoma, transformed retinoblasts and a number of other human and rodent cell lines during apoptosis (references in[62]). DNA sequencing revealed this protein to be homologous the yeast Apg5 gene. To date however, a functional link between ASP/Apg5 expression and cell death has not been established. Most recently, beclin-1 was the first gene described to induce autophagocytosis in mammalian cells.[63,64] Beclin 1 is a bcl-2-interacting protein with structural similarity to the yeast autophagy gene apg6/vps30. It was found to be expressed ubiquitously at high levels in normal breast epithelia, but mono-allelically deleted in 40–75% of sporadic human breast cancers and ovarian cancers. Beclin-1 promoted autophagy in yeast and in human MCF-7 breast carcinoma cells; beclin-1 induced formation of autophagic vacuoles was prevented by 3-MA. The autophagocytosis-promoting activity of beclin 1 in MCF-7 cells was associated with inhibition of MCF-7 cell proliferation, *in vitro* clonigenicity and tumorigenesis in nude mice. In these studies, no evidence for an enhanced rate of cell death in MCF-7.beclin1 clones was found using trypanblue exclusion as an indicator of cell viability.[64] However, previous studies of our own with MCF-7 cells revealed that the manifestation autophagic cell death by nuclear condensation/fragmentation was neither associated with release of cytoplasmic enzymes into the culture medium nor with a significant trypanblue staining of dead cells, thus matching with the metabolic requirements of autophagocytosis.[39,45] These observations suggest that autophagic cell death – like apoptosis – at least in early stages is not associated with loss of cell membrane integrity. Thus, whether beclin-1 also might induce cell death cannot be excluded yet. Downstream of Apg-genes act TOR (target of rapamycin) and p70S6-kinase; the TOR/p70S6-kinase pathway plays an important role in balancing anabolic and katabolic states of cells.[45,61,65–68] Hypophosphorylated p70S6-kinase promotes detachment of ribosomes from endoplasmic reticulum; this is considered to be one of the initial molecular events in sequestration (for review:[39,45,46]). As outlined above, the sequestration step provides an important regulatory link between autophagocytosis and cell suicide and therefore, the TOR/p70S6-kinase pathway appears to be a promising target for studying the interaction between autophagocytosis and cell death.

Studies by Kuchino *et al*.[52,53] on the RAS-signalling pathway provided the first clear evidence for molecular interactions between autophagy and programmed cell death in mammalian cells. It has been shown that the expression of oncogenically mutated *ras* gene in human glioma and gastric cancer cell lines induces cell death including autophagocytosis. The nuclei remained relatively well-preserved and were negative for TUNEL staining,[52,53] thus matching with the general morphological features of type II PCD (cf Table 1). The oncogenic Ras-induced cell death was dependent on the activity of phosphatidylinositol-3-kinase (PI3-K), a physiological downstream effector of Ras.[53] A seemingly paradox was that PI3-K activity is required for both, induction of autophagocytosis but also for S6-phosphorylation of the ribosomal protein S6 and consequently, block of autophagocytosis (see above). However, recent studies on human cancer cells revealed that distinct classes of phosphatidylinositol-3-kinases act in opposite directions in the pathways signalling for sequestration.[54] Thus, the class III PI3-K product PtdIns(3)P is required for sequestration; formation of PtdIns(3)P as well as of autophagosomes was found to be inhibited by 3-MA, wortmannin and LY294002.[46,54] On the other hand, increasing the class I PI3-K products PtdIns(3,4)P and PtdIns(3,4,5)P inhibits macropautophagocytosis and favours protein synthesis, cell proliferation and cell survival

(Figure 2).[46,54,67] Furthermore, Ras-induced cell death occurred in the absence of caspase activation, it did not require wt-p53 activity and was not inhibitable by the anti-apoptotic Bcl-2 protein.[53] Notably, the functional effector machinery for the execution of apoptosis could be activated in the Ras-transformed cells by appropriate signals, demonstrating that the manifestation of autophagic cell death does not simply reflect defective apoptosis.[53] Thus, these studies strongly suggest that autophagic cell death may be assigned to the caspase-independent type(s) of programmed cell death including apoptosis, as recently reviewed elsewhere.[2,52,69,70] Likewise, studies on isolated neurons revealed that the manifestation of autophagic cell death may be controlled upstream of caspase cascades, but downstream of JNK/p38 (after NGF-withdrawal) and p53 (after cytosine arabinoside).[40] These studies also suggested that the same apoptotic signals that target mitochondria also activate autophagy. Once activated, autophagy may mediate caspase-independent neuronal cell death.[40]

Taken together, although a few molecular features (apg-genes, Ras, PI3-K) may be assigned to the morphological manifestation of autophagic cell death, the mechanisms of initiation and execution of this type of programmed cell death are still enigmatic. However, the few tesseras on molecular interactions between autophagy and cell death obtained so far support the concept that both processes share common signalling pathways. The apg-gene family and TOR/p70S6 kinase pathway provide most likely candidates. The RAS–signalling in autophagic cell death, for instance might interact with this pathway by crosstalk via PI3-kinases. Furthermore, as outlined above apoptosis and autophagic cell death are not mutually exclusive phenomena, but may occur in the same cell. In support and extension of the morphological observations, recent high-throughput proteom analyses revealed evidence that autophagic death of tamoxifen treated MCF-7 cells and CD95-induced apoptosis in Jurkat cells shared some commonalities as exemplified by the cell's stress response (translocation of heat shock proteins).[12,71]

Differences and commonalities of autophagocytosis and phagocytosis

Autophagocytosis and phagocytosis share a dynamic reorganisation in the structure and composition of membranes.[5,45,46,72] In case of phagocytosis, ligation of external particles such as apoptotic bodies to the phagocyte membrane initiates its reorganisation; the ligation appears to be driven by surface tags of apoptotic bodies or by release of soluble factors from dying cells targeted at receptors of the phagocytes.[5,46,72,73] Engulfment may be considered functionally equivalent to the sequestration step during autophagocytosis; as initial events both precede (auto)phagosome formation. Biochemically, these steps share their requirement for actin.[5,45,46,72] Notably, the size limits of particles for being processed through either pathway are in the same range, namely 300–900 nm in diameter.[46,72,73] Like the tagging of apoptotic cells, the targeting of mitochondria, peroxisomes, ER-membranes and cytosolic constituents for sequestration appears closely regulated. Macroautophagy can operate with selectivity for certain subcellular structures over others, but also in a largely nonselective fashion (for review:[39,45,46]). For instance during regression of chemically induced rat liver hypertrophy, selective autophagic elimination of either smooth endoplasmic reticulum or peroxisomes (pexophagy) has been observed; mitotic chromosomes[43] and damaged mitochondria can be eliminated by the same way (for review:[39,45,46]). In general, the underlying mechanisms for selection or exclusion of cell components for/from autophagy are poorly understood. Future studies will have to address the underlying molecular events and their link to cell death signalling pathways.

Remarkably, a number of observations suggested that the autophagic type of cell death ensues independent of caspases.[52,69,70] In apoptosis, the caspase cascades provide a powerful tool to mediate diverse pro-apoptotic signals to a 'final common pathway'; most if not all the prominent morphological features of apoptosis as originally described are caspase-dependent.[2] From a teleological point of view, apoptosis is designed to delete cells from tissues rapidly; the clearance of apoptotic cell residues from tissues is facilitated by tagging them for phagocytosis[5] as well as by volume reduction (condensation/fragmentation) being appropriate for phagocytosis (300–900 nm). What would be the advantage for activating an autophagic type of cell suicide? Hypothetically, self-digestion preceding suicide might reduce the functional load imposed on the surviving cells by phagocytosis and break down of huge amounts of dead cells as necessary in remodelling tissues; thereby, a rapid elimination of cells would be facilitated and would help to prevent inflammatory and immunological responses.[5] In addition, soluable molecules resulting from autophagic breakdown might be recycled by other mechanisms such as pinocytosis.

The final (post mortem) degradation of apoptotic bodies as compared to the final stages of autophagocytosis deserves a comment. For instance, in the liver *in vivo* the morphological (incl. size) and histochemical features of phagocytosed apoptotic bodies closely resemble those of autophagic vacuoles.[74,75] Thus, the final lysis and reutilisation of the digested material seems very likely not to differ significantly except its duration: in the liver *in vivo*, the half-life time for the clearance of apoptotic bodies was found to be about 120 min,[75] that of autophagic vacuoles ranged from 5–45 min, depending on the material subjected for degradation.[76] From a practical point of view, the similarities in the post mortem appearance of apoptotic bodies and autophagic vacuoles are of importance as the unequivocal identification and quantitative analysis of either phenomenon can be affected. For instance, in *in vivo* studies on small intestinal crypts of normal mice and Crocker mouse ascites tumours treated with cytostatic drugs, phagocytosed apoptotic bodies have been mistaken for autophagic vacuoles.[77] However, apoptotic bodies (ABs) can be discriminated from autophagic vacuoles (AVs) based upon the chromatin residues present in ABs, but usually not in AVs; electronmicroscopy and/or specific stains to visualise DNA revealed to be most helpful tools as reviewed previously.[75] Finally, in cultured

cells cytoplasmic vacuolisation is widely observed, but this type of vacuolisation is considered to be distinct from that consequently to autophagy (for review:[78]). Taken together, these phenomena should be taken into account and appropriate techniques should be used to verify gross morphological observations.

Lysosomes in cell death

Early after discovery, lysosomes ('lytic bodies') have been associated with necrosis ensuing after cell damage, but are not generally considered as its primary cause.[79–82] For instance, a close time course study on chemical hypoxia induced cell damage in cultured hepatocytes, namely ATP-deletion, bleb formation with cellular swelling, onset of a mitochondrial permeability transition, disintegration of lysosomes, plasma membrane failure from bleb rupture, and cell death has been published more recently.[82] This study suggests that the release of hydrolytic enzymes from lysosomes may be the final event causing lysis of the membrane and irreversible loss of viability.[82]

However, activation of lysosomal enzymes is not restricted to the necrotic type of cell death. Thus, a number of lysosomotropic agents has been described to induce apoptosis (Figure 3).[83–90] The potency of lysosomal enzymes to trigger apoptosis revealed these organelles as potential targets for increasing a cell's sensitivity for photodynamic therapy, for instance by facilitating oxidative stress via intralysosomal fenton-like reactions.[83,91–93] In general, the magnitude of lysosomal rupture and consequently, the amount of hydrolytic enzymes released into the cytosol may induce either reparable sublethal damage, apoptosis, or necrosis;[86,88,94,95] the dose-dependency of causing either apoptosis or necrosis is exemplified by the lysosomotropic agent MSDH (apoptosis $\leqslant 50\ \mu$M *vs* necrosis $\geqslant 75\ \mu$M; Figure 3).[88] The decision between necrosis or apoptosis may also depend upon the organelle being targeted primarily as shown in murine leukaemia L1210 cells treated with the photosensitising agent chlorin e6 triacetoxymethyl ester: a low dose targeted mitochondria and triggered apoptosis, whereas a higher dose targeted lysosomal membranes with cell death likely occurring via a necrotic process.[95]

The lysosomal cystein proteases, cathepsins, have been implicated in the activation of caspases and apoptosis (Figure 3[89,90,96,97]). For instance, studies on cultured fibroblasts and cardiomyocytes revealed that lysosomal destabilization (measured as release of cathepsin D) precedes release of Cytochrome *c*, loss of mitochondrial membrane potential and morphologic manifestation of apoptosis.[89,90] Pepstatin A, an inhibitor of cathepsin D, was found to inhibit caspase-3-like proteolytic activity and to prevent apoptosis in several experimental settings.[89,90,98,99] Notably, two p53 DNA-binding sites located in the cathepsin D-promoter have been found to specifically bind to p53 protein *in vitro* and appeared to mediate transactivation during p53-dependent apoptosis.[99] Moreover, high levels of cathepsin D antisense RNA protected HeLa cells from interferon-gamma and Fas/APO-1-induced death.[100] In transgenic models overexpression of cathepsin D induced or sensitised HeLa and PC12 cells to apoptosis upon serum deprivation.[101] Other lysosomal cysteine proteases such as cathepsin B,C,L have been implicated in caspase activation as well.[97,102,103] In particular, in cell free systems purified cathepsin B has been found to directly cleave caspase zymogens: it readily cleaved

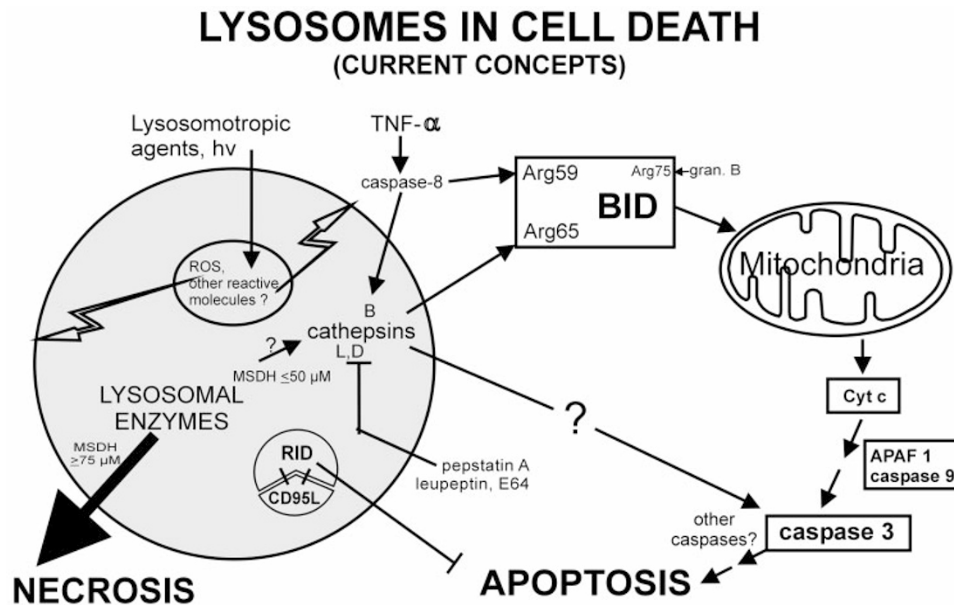

Figure 3 Potential roles of lysosomes in cell death. Summary of current concepts on the role of lysosomes for the induction of cell death, see text for explanation and references. Note: simplified as not distinguished between different stages of lysosome maturation. Lysosomotropic agents: α-tocopheryl succinate[84]; 9-Acetoxy-2,7,12,17-tetrakis-(beta-methoxyethyl)-porphycene (ATMPn)[87]; O-methyl-serine dodecyl-amide hydrochloride (MSDH)[88]; 5,8-dihydroxy-1,4-naphthoquinone[89]; chlorin e6 triacetoxymethylester (CAME)[95]; polyamine oxidase inhibitor MDL-72,527[118]; imidazo-acridinone C1311[119]; retinol[120]; Necrosis: [86–88,94,95]

procaspase-11 and 1; procaspases 2, 6, 7 and 14 revealed to be weak, procaspase 3 a very poor and finally, procaspase 12 to be no substrate for cathepsin B.[102] However, the physiological relevance of a direct physico-chemical interaction between cathepsin B and caspases has been challenged. Thus, Salvesen *et al*.[104] most recently found no evidence for a direct role of lysosomal proteases in caspase activation. Rather, proteases that have leaked from lysosomes appear to cleave BID at Arg65 and thus, caspase activation may ensue via the mitochondrial pathway (Figure 3). The authors proposed that BID acts as a general sensor of proteolysis by endopeptidases and that this pathway enables cells to respond to adventitious and potentially harmful proteolysis by executing the apoptotic suicide.[104] Likewise, cathepsin B has been found to contribute to TNF-α-mediated hepatocyte apoptosis by promoting mitochondrial release of Cytochrome *c* (Figure 3).[105] The lysosomal cystein proteases, however, seem not to act exclusively via the mitochondrial Cytochrome *c*/APAF-1/caspase-9 cascade as evidence for a lysosomal-mediated activation of caspase-3 by a distinct pathway has been provided (Figure 3).[106] Taken together, the specific roles of lysosomal cysteine proteases in the activation of caspases await elucidation. It should be also noted, that inhibition of cathepsins in neuronal cells and in primary hepatocyte cultures has been found to result in induction rather than inhibition of apoptosis.[107,108] Finally, the plethora of lysosomal enzymes include nucleases and indeed, lysosomal endonucleases have been described giving raise for DNA fragmentation considered typical of apoptosis, using photo-oxidative damage to destabilise lysosomal membranes.[85,86]

Lysosomes have also been found to be involved in the control of CD95L presentation at the cell surface and thereby, of apoptosis. Thus, newly synthesised CD95L is stored in specialised secretory lysosomes in CD4+ and CD8+ T cells as well as natural killer cells; polarised degranulation controls the delivery of CD95L to the cell surface and eventually apoptosis.[109] Likewise, in adenovirus infected cells the adenovirus RID (receptor internalisation and degradation) protein complex, mediates internalisation of cell-surface CD95 and its destruction inside lysosomes (Figure 3). Removal of CD95 from the surface of adenovirus-infected cells expressing RID may allow infected cells to resist CD95-mediated cell death and thus promote their survival.[110] *In vivo*, promotion of cell survival has been observed in transgenic mice: congenital deficiency of lysosomal beta-glucuronidase results in prolongation of CrmA expression and thereby, inhibition of apoptosis.[111] On the other hand, it should be reminded that in many cell types lysosomes secrete their content after fusion with the plasma membrane.[112] Thus, secretory lysosomes of cytotoxic lymphocytes contain essential apoptotic molecules to eliminate virus-infected cells, namely the membranolytic perforin, and the serine protease granzyme B; the eventual cell death induced by granzyme B was found to be caspase-independent.[113] Recently, dipeptidyl peptidase I, a lysosomal cysteine protease, has been found to be essential in the *in vivo* processing and activation of granzymes A and B.[114]

In conclusion, for many years lysosomal enzymes have been known to be involved (1) in necrotic type of cell lysis and, (2) in digestion of apoptotic cell residues upon their phagocytosis by vital neighbours, conceivably involving the whole plethora of lysosomal enzymes. More recently, accumulating evidence strongly suggests that lysosomal cysteine proteases may trigger preparatory steps of cell suicide; the underlying molecular mechanisms, however, are not yet elucidated. Nevertheless, diverse or relatively unspecific signals such as photodamage or lysosomotropic agents may be mediated to the specific enzyme cascades leading to coordinated final self-destruction of cells. The apparent role of lysosomes in programmed cell death adds support to the view that lysosomes are not simply a 'garbage-disposal-unit' as outlined recently by Luzio *et al*.[55] Furthermore, the role of lysosomal system in programmed cell death deserves attention in view of their role in senescence and storage diseases.[115]

Conclusions

Programmed cell death (PCD) is an essential phenomenon in normal development and adulthood of multicellular organisms. Cells use different ways for active self-destruction, with the morphology ranging from apoptosis to autophagic cell death. Autophagic cell death appears to be activated when massive removal of cells or cytoplasm is demanded, for instance by developmental programmes. Autophagy preceeding cell death may also reflect a cell's adaptive response to sublethal (non-necrotic) conditions such as nutrient/growth factor deprivation or cell damage by cytotoxic drugs, hypoxia etc. A functional link is provided by a number of studies showing that 3-methyladenine inhibits both, formation of autophagosomes and the manifestation of cell death (nuclear collapse). However, so far no causative relationship between autophagocytosis and eventual cell death has been established. Nevertheless, some molecular features such as Ras-signalling, PI3-kinases and the autophagocytosis genes apg5/ASP and apg6/vps30 (beclin-1) might be assigned to pathways leading to the morphological appearance of autophagic cell death and provide promising targets for further studies. Furthermore, apoptosis and autophagic cell death are not mutually exclusive phenomena, they may occur simultaneously in tissues or even, conjointly in the same cell; both processes may end, if cell residues are not phagocytosed, in secondary necrosis. It should be emphasized, that programmed cell death appears to be highly conserved during evolution as it occurs in unicellular organisms,[116] in the green algae Volvox spec regulating the germ-soma dichotomy,[117] the slime mold *Dicytostelium discoideum*[15,16] and, last but not least, in plants (see a series of reviews published in Cell Death and Differentiation 4(8), 1997). Golstein and coworkers raised the hypothesis that a single-core mechanism of PCD that may have emerged before the postulated multiple emergences of multicellularity has been raised.[15,16] According to this hypothesis, the phenotypic variations of PCD would result from differences in enzymatic equipment and mechanical constraints adjusted to the given biological conditions.

Furthermore, there is sufficient evidence to suggest that lysosomes are important mediators of programmed cell

death. Proteases released from the lysosomal compartment may trigger initiating events of apoptosis. Lysosomes may also be rate-limiting for the delivery of death receptors to the cell surface, thereby modulating the sensitivity of cells to external ligands. Taken together, these observations strongly suggest that lysosomes, like the mitochondria and the endoplasmatic reticulum, may play an important part in apoptosis signalling.

Acknowledgements

I would like to thank Rolf Schulte-Hermann for critical reading of the manuscript.

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