The evolution of cell death programs as prerequisites of multicellularity

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Received 4 September 2002; accepted in revised form 2 December 2002

In memoriam of my father, Hans Krupitza

Abstract

One of the hallmarks of multicellularity is that the individual cellular fate is sacrificed for the benefit of a higher order of life—the organism. The accidental death of cells in a multicellular organism results in swelling and membrane-rupture and inevitably spills cell contents into the surrounding tissue with deleterious effects for the organism. To avoid this form of necrotic death the cells of metazoans have developed complex self-destruction mechanisms, collectively called programmed cell death, which see to an orderly removal of superfluous cells. Since evolution never invents new genes but plays variations on old themes by DNA mutations, it is not surprising, that some of the genes involved in metazoan death pathways apparently have evolved from homologues in unicellular organisms, where they originally had different functions. Interestingly some unicellular protozoans have developed a primitive form of non-necrotic cell death themselves, which could mean that the idea of an altruistic death for the benefit of genetically identical cells predated the invention of multicellularity. The cell death pathways of protozoans, however, show no homology to those in metazoans, where several death pathways seem to have evolved in parallel. Mitochondria stands at the beginning of several death pathways and also determines, whether a cell has sufficient energy to complete a death program. However, the endosymbiotic bacterial ancestors of mitochondria are unlikely to have contributed to the recent mitochondrial death machinery and therefore, these components may derive from mutated eukaryotic precursors and might have invaded the respective mitochondrial compartments. Although there is no direct evidence, it seems that the prokaryotic–eukaryotic symbiosis created the space necessary for sophisticated death mechanisms on command, which in their distinct forms are major factors for the evolution of multicellular organisms.

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Keywords: Apoptosis; Necrosis; ATP; Mitochondria; Evolution

Abbreviations: ADP, adenosine diphosphate; AIDS, acquired immune deficiency syndrome; AIF, apoptosis inducing factor; ANT, adenine nucleotide translocator; ATP, adenosine triphosphate; CAD, caspase-activated DNase; caspase, cysteine aspase; dATP, deoxy-adenosine triphosphate; DISC, death-inducing signaling complex; ΔΨm, mitochondrial membrane potential; ER, endoplasmatic reticulum; Fas, Fas-L, Fas-ligand; FK506, immuno-suppressant isolated from Streptomyces sp.; IAP, inhibitor of apoptosis; mTOR, mammalian target of rapamycin; NAD, nicotinamide adenine dinucleotide; PT, pore transition; ROS, reactive oxygen species; TIR, toll-like interleukin-receptor domain; TNFα, tumor necrosis factor α; TRAIL, TNF-related apoptosis-inducing ligand; z-VAD-fmk, benzyloxycarbonyl Val-Ala-Asp-fluoromethylketone, a specific inhibitor of caspases

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doi:10.1016/S1383-5742(02)00110-2
1. Introduction; life and the meaning of death

The term “life” describes a combination of phenotypes such as metabolic activity, its restriction to complex structures, growth, and the potential to identically self-reproduce. When we limit our observations to prokaryotes “death”, of course, does not occur in this description. In these primitive organisms death only seems to be a consequence of environmental conditions that are not compatible with the biochemistry and metabolism that accompanies “life”. In eukaryotes this accidental cell death constitutes the phenotype called necrosis. In contrast, multicellular organisms developed complex cell suicide mechanisms to circumvent necrosis and also in unicellular eukaryotes non-necrotic cell death was described [1,2]. Prokaryotes seem to lack those homologous components required to die in orchestrated fashion although the commitment to suicide was observed during fruitbody formation of Myxobacteria [3]. A few unicellular eukaryotes possess a primitive apoptotic outfit [2,4–7] which is however, not regulated on program. In general, there is an apparent lack of homologies (orthologues) between unicellular death modules and those of recent metazoans, and therefore, a secondary death machinery might have been acquired entirely independent from higher organisms. In this respect, the term apoptosis just defines the mechanism(s) leading to the phenotype of resorptive self-destruction.

In extension, “programmed cell death” includes the invention of intrinsic and extrinsic trigger factors, as part of an integrated genetically determined process, that becomes activated when appropriate [8–11].

Once single cells started to organize into multicellular forms it was apparently advantageous to invent a cell death program as a means to shape structures and to balance this complexity. This implies that the individual cellular fate was sacrificed for the benefit of a higher order of life, and seems to evidence the existence of a principal archaic hierarchy of being. However, this point of view neglects the fact that evolution, as we know since Charles Darwin, does not “invent” new genes with specific functions but varies and adapts the existing outfit by DNA-mutations and this genetic plasticity allows to develop new shapes and advanced functionality. Thus, some components that gradually gained functions for self destruction already existed in unicellular eukaryotes, albeit in different contexts. But even cell death mechanisms might have existed in ancestral protozoans, because in a few contemporary unicellular parasites and in Dictyostelium discoideum we find cell death phenotypes reminiscent of apoptosis [2]. These mechanisms not only allow differentiation into spores or cysts but enable the survival of a colony in adverse conditions and hence it seems that these mechanisms were mandatory to find niches for multicellularity [2]. In this respect it is of note that eukaryotic AIF could be tracked down to some of the diverse group of the archaea, which are the assumed ancestors of today’s eukaryotes [13]. Also paracaspases which are found in Dictyostelium and metazoans, and metacaspases which are found in protozoans, fungi and plants, indicate a common ancestry [12]. However, in unicellular eukaryotes these cysteine proteases seem to serve in signal transduction and have acquired their new function in apoptosis later on [13].

The death effectors that operate in protozoans are still enigmatic and furthermore there exist also protozoans that cannot undergo an apoptosis-like phenotype. It has to await clarification whether the death machinery was lost by reductive evolution, or whether those species without apparent self destruction mechanisms represent an early phylogenic life form. Alternatively, the death components in parasitic unicellular eukaryotes might have developed during the host-defense evolution. Notably, even in unicellular eukaryotes “apoptotic” features have been described [3,14,15] and even examples for shared homologies exist: the TIR [13,16] is homologous in animals, plants, and bacteria. Toll receptors play a role in the immune defense against parasites and can elicit apoptosis. Furthermore, bacterial serine protease HtrA shows homology with mammalian HtrA2 [17]. However, bacteria require HtrA to tolerate thermal, osmotic and oxidative stress [18] and the chaperone function of the bacterial homologue changed to an IAP-inhibitor, which is a protein that counteracts caspase activity in eukaryotes [19,20] in analogy to Smac [21–23].

As the driving force, increased or new environmental pressure, might have urged for different forms of cell death, which were accomplished over time. We cannot track back the succession of mutations that were necessary to evolve cell death programs
mandatory for the evolution of higher order life forms. However, we know from pathologies such as cancer or Alzheimer’s disease, that loss- or gain-of-function mutations of apoptosis genes destroy this complexity [24].

2. Necrosis—a threat to the higher order

Necrotic (accidental) cell death results from a variety of stresses, such as extreme physicochemical injury (radicals, radiation, temperature, toxic trauma, etc.), osmotic imbalance, abrupt anoxia, energy deprivation (sudden shortage of nutrients such as glucose), but also when apoptotic execution pathways are blocked after physiological apoptosis-induction. Hence, necrosis is always the outcome of severe acute insults that cause almost instantaneous membrane depolarization and disruption. The destruction of permeability barriers (ΔΨ Collapse) upon stress and the resulting collapse of separated biochemical regulatory cycles that maintain life-processes, is an inadequate mechanism for metazoan cells to evade life, because of the threat to the superior hierarchy. Necrotic spillage of cellular components, which are otherwise stored away in subcellular compartments, would induce inflammatory reactions that affect neighboring cells which in consequence become necrotic themselves, resulting in a constant spreading of necrotic somatic areas. In general, inflammatory responses are physiological adaptations of the immune system in pathogen defense, but cell fluids that are normally not exposed also elicit this response. Thus, necrosis is an inadequate means to maintain homeostasis, because it can lead to auto-immune reactions. To allow for proper, (stress-triggered) escape from life without damaging considerable tissue areas, it requires a mechanism which is non-toxic to the cell-neighborhood. The immune system developed highly sophisticated apoptotic host-defense mechanisms against cells which are genetically altered- or “non-self”, thereby defending off potentially dangerous cells and contributing to homeostasis. Moreover, “activation induced cell death” evolved to down-modulate the immune response, which is regulated by death ligand-death receptor interactions, and to limit the expansion of cells responding to a particular antigen. The fundamentals of these apoptotic defense systems, however, did not arise in the cells of the immune system, but earlier in evolution in less specialized cells favoring innocuous death as a prerequisite for the development and maintenance of multicellular aggregations. Therefore, ancient death-components are highly homologous from the primitive nematode worm Caenorhabditis elegans to the arthropod Drosophila to vertebrates, and the components increased in number and complexity throughout phylogenic evolution [25].

Below certain threshold limits of noxious insults cells vanish through apoptosis or may survive due to cellular de-toxification, DNA-repair, or emergency mechanisms. However, upon severe and immediate impact the damage is either too massive to be repaired or time is too short to allow for an apoptotic response. In either case, cells burst by a phenotype called primary necrosis. Depending on the extent of the damage caused by stress factors, and whether or not energy supply is sufficient for the completion of physiological emergency programs cells may still become necrotic due to energy depletion, although the apoptotic machinery might have been already sparked [26]. This constitutes the phenotype called secondary necrosis. Therefore, cells that were exposed to necrotic stresses, may also exhibit apoptotic early stage phenotypes [27–29].

Interestingly, a component of a DNA proof-reading and repair mechanism, poly(ADP-ribose) polymerase (PARP) [30,31], is the to date only known element that is sensitizing a cell for necrosis [32,33] but does not modulate apoptosis [34–36]. PARP utilizes NAD as a substrate to synthesize and transfer poly(ADP-ribose) upon prolonged PARP-activation the NAD-pool and the ATP-level drop dramatically and cells die [37–40]. PARP−/− cells, that do not consume NAD in response to DNA-strand breaks, and therefore exhibit improved ATP maintenance, are less sensitive towards necrotic stimuli, and PARP−/− mice do not suffer from cerebral ischemia [33]. Hence, ATP is considered to be a switch between apoptosis and necrosis [41–43]. However, recent discussion considers that ischemic brain damage is not only based on necrotic but also on apoptotic cell death of the adjacent tissue [44], and furthermore, that ischemic cell death is a separate type of apoptosis [45]. Therefore, the role of PARP in necrosis (but not in apoptosis) needs further clarification. Nevertheless, based on compelling
3. The evolution of programmed cell death

Death programs are tightly linked to eukaryotic–bacterial endosymbiont co-evolution. Two distinct evolutions by bacterial endosymbiosis gave rise to eukaryotic realms. Firstly, alpha-proteobacteria developing to the mitochondria of all eukaryotic cells and secondly, cyanobacteria that changed to chloroplasts in plant evolution. Although we do not have evidence today that more and diverse bacterial endosymbiosis had evolved, we can still take it as almost certain that this had happened but was eradicated by competition or catastrophe. The amoeba Palomyxa palustris hosts endosymbiotic aerobic bacteria which metabolically substitute for the lacking mitochondria [2]. This evidences that convergent endosymbiont evolution is still going on.

Many regulators of apoptosis are located in the mitochondrial or in the mitochondrial inter-membrane space (caspases 2, 3, and 9 [46], cytochrome c [47], Bcl-2 [48], Bcl-X [49,50], Bad [51], Bak [52], Bak [53], Bim [54], Smac [55], HtrA2 [22], AIF [56,57]). However, we do not find these or homologous components (except HtrA) in contemporary bacteria. The question arises: Did the aerobic bacterial predecessors of mitochondria possess apoptotic regulators that gradually vanished in modern bacteria and other prokaryotes? Usually systems increase in complexity during phylogenesis and, once mechanisms or structures got lost by reductive evolution, it has not been observed so far in biological systems that identical parts or pieces were re-introduced later on by homologous structures or components, but only by analogous ones due to secondary convergent evolution (i.e. fish fins versus dolphin fins).

A few hypotheses were brought up, to explain how the apoptotic outfit might have evolved in metazoans. One hypothesis speculates [46] that the metazoan apoptotic machinery “invented” the mitochondrial membrane interspace during evolution, because this compartment is a well buffered niche and perfect to safely store away life-threatening molecules. Two arguments are supporting this hypothesis:

1. Death effectors (i.e. caspases 2, 9, and 3) are only active at acidic pH but not in the mitochondrial, well buffered, inter-membrane compartment. Thus, apoptogenic components would have developed by eukaryotes after the prokaryotic mitochondrial ancestor got manifested as endosymbiont.

2. Yeast and other unicellulars, which also contain mitochondria lack the apoptogenic death components known from metazoans. Thus, mitochondria are not the source of death components. However, it was shown that Saccharomyces cerevisiae [58–60], Schizosaccharomyces pombe [61], trypanosomes [1,6], Leishmania [62] and a few other unicellular eukaryotes [63,64], can undergo apoptosis-like cell death upon exposure to ROS [65,66].

Another hypothesis proposes that the evolution of mechanisms, which provoke apoptosis-like phenotypes in a number of unicellular eukaryotes, have developed by the selective pressure of limited nutritional supply. This would eradicate affected genomes (turmoiled by genotoxic stress) in favor of intact ones without poisoning limited nutritional resources by necrotic spillage [26,58,59,61,65]. This hypothesis suggests that unicellular eukaryotes, single individuals and virtually un-connected to each other, may have invented a strategy for the benefit of the whole species, that is reminiscent of the complex cellular interrelations and adaptations of metazoan tissue development. In retrospect, this makes the hypothetical species look “altruistic”. In fact, slime molds, in which AIF acts as apoptotic factor, can do both: live as single amoeboïd individuals but can also aggregate and behave like a multicellular organism in the search for nutrients [4]. Another “altruistic” hypothesis was put forward [16], which speculates that parasitic infections, that would have been lethal for protozoans, might stand at the very beginning of the development of apoptogenic mechanisms. In this hypothesis, the altruistic suicide of the infected individual (the individual cell would die anyway) would also destroy the parasite, which could otherwise move on to the next, presumably closely related protozoan. Thereby it preserves
the rest of the population and this “altruism” exerts a selective advantage for the species. These hypotheses have in common, that a death machinery gradually developed from already existing polypeptides by successive mutational events. The new gene functions benefited an assumed ancestral population of unicellular individuals, which included the option to get on the evolutionary way to invent multicellular complexity in the future. This may exemplify, that a primitive suicide machinery was the prerequisite to start metazoan evolution, which is widely accepted, yet has to be proven.

Regardless whether the basic apoptotic machinery emerged in the one way or the other, within the framework of the ancestral metazoan self-destruction “organelle” provided by the endosymbiont, distinct multi-component death mechanisms emerged.

4. Distinct genetic programs to terminate life

Several morphologically and biochemically discernible forms of programmed cell death have been described.

4.1. Type I cell death

4.1.1. Caspase-dependent apoptosis

This is a physiological process of cell suicide eliminating superfluous or unwanted cells. Apoptotic cell death involves the orchestrated action of catabolic enzymes (proteases, nucleases) within the limits of intact plasma membranes and is accompanied by a characteristic change of nuclear morphology (see Fig. 1) and chromatin biochemistry (stepwise DNA degradation [67]). Specific cysteine proteases (caspases) cleave their targets after aspartic acid residues and catalyze a highly selective pattern of protein degradation. Moreover, cellular organelles remain morphologically intact (biochemically only subtle changes of organelles become manifest in dying cells such as membrane permeabilization and partial protein degradation), whereas cells shrink and reduce intracellular potassium level [68,69]. In the aftermath of apoptosis, because the remaining material is in relative small units, they are readily phagoytosed by unaffected neighboring cells.

4.1.2. Caspase-independent apoptosis

Currently we know that the execution of apoptotic chromatin degradation can be achieved not only by executioner caspases 3, 6, and 7 and their effector CAD [70,71], which generates 180bp DNA fragments and multiples thereof, but also by AIF in a caspase-independent fashion. AIF activates a nuclear DNAse [72] which cuts genomic DNA into 50 kb fragments giving rise to a distinct nucleo-morphological phenotype of chromatin condensation, which is called stage I [73]. Whereas CAD generates the phenotype of stage II chromatin condensation (see Fig. 1). The two mechanisms of apoptosis execution exist together and may cooperate. It needs to be determined whether either of the pathways can be triggered separately depending on the cell type, the context, or the stimulus. The question why two apoptosis pathways evolved in parallel remains open. We find numerous examples showing convergent evolutions of organs and structures, which in contrast to homologies (orthologues), are termed analogies (paralogues). Thus, nature might as well have invented self-destructing mechanisms a second or third time simultaneously or in parallel, utilizing the same frame structures such as the mitochondrial inter-membrane space (because of advantage), but developing distinct functional components (because no homologies of apoptotic molecules between yeast and metazoans were found to date). Throughout cell death evolution the proapoptotic mammalian factor Smac [55] and the fruitfly factors Reaper, Grim and Hid [74] function in a similar way by inhibiting IAPs, but they are not homologous.

4.2. Type II cell death

Cell death by autophagy, or type II cell death, involves lysosomes. Upon induction, cytoplasmic material and relevant subcellular organelles such as ER and/or Golgi become sequestered and parts of their membranes assemble to autophagosomes. These further integrate with lysosomes and build up the autophagic vacuoles, which can be visualized by staining with monodansylcadaverine [75] (see Fig. 1). Even mitochondria become degraded despite a few required for ATP-supply. Furthermore and in contrast to apoptosis, the cytoskeleton remains intact (see Fig. 1). This is a fundamental difference to apoptosis.
Fig. 1. Doxorubicin simultaneously induces apoptosis, necrosis and autophagosome formation in SKBR-3 breast cancer cells. SKBR-3 cells were triple-stained with Hoechst 33258 (2.5 μg/ml), propidium iodide (1 μg/ml) and mono-dansylcadaverine (1:3000 dilution of a saturated 50% ethanol solution) and photographed with a 400-fold magnification using a Zeiss Axiovert microscope connected to a UV-lamp and a Zeiss DAPI filter no. 02 (left side panels; H-P-C). Right side panels show the identical microscopical frames photographed with phase contrast (P-C). Panels (A): Control cells were treated with solvent and exhibit normal nuclear Hoechst 33258 chromatin staining (dark blue). (a) Shows a cell undergoing spontaneous apoptosis at an early stage (bright blue nuclear staining due to chromatin condensation). Panels (B): Cells were treated with 5 ng/ml doxorubicin for 96 h. (a) Points at an early apoptotic cell nucleus (bright blue). Exposure to doxorubicin clearly increases the apoptosis rate. Panels (C): Cells were treated with 40 ng/ml doxorubicin. (a) Shows a growing number of late apoptotic cells, which are in the process of losing membrane integrity. Due to intrusion of propidium iodide, which causes the merging of the colors red and blue, the chromatin stains pink-white. Panels (D1): Arrow (n) points at a necrotic cell. Due to disrupted membranes, as a hallmark of necrosis, propidium iodide intrudes resulting in evenly pink-white nuclear staining. Condensed chromatin, which is typical for apoptosis does not occur in necrotic cells. Arrow (a) points at an apoptotic cell and arrow (au) at a cell which exhibits autophagosome formation detected by mono-dansylcadaverine (blue cytoplasmic spots). The nuclear morphology of this cell (au) is still intact and prevents intrusion of propidium iodide and hence, the chromatin is only stained by Hoechst 33258 (blue nucleus). Therefore, the cell is presumably alive, or considered as "not dead yet", and might recover upon drug removal. However, the cell shown in panels (D2) by arrow (au), which also forms autophagosomes, but lacks nuclear staining, has reached an advanced stage of autophagy and is certainly not viable any more. The remaining cell structure (see phase contrast; D2) suggests that cytoplasmic structure-proteins are still preserved as a hallmark of type II cell death. By which mechanism the chromatin vanishes remains enigmatic. In panels (D1) and (D2) cells were treated with 100 ng/ml doxorubicin and the images were additionally magnified two-fold and brightened by Adobe Photoshop program for improved demonstration.
Table 1
Distinct mechanisms causing cell demise

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Autophagy</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiological/pathological features</td>
<td>Induced by various physiological and noxious stimuli, and during development</td>
<td>Induced upon severe stress, toxic exposure and/or ATP-depletion</td>
</tr>
<tr>
<td>No inflammatory response</td>
<td>No inflammatory response</td>
<td>Inflammatory response</td>
</tr>
<tr>
<td>Affection of individual cells</td>
<td>Affection of individual cells</td>
<td>Massive affection of tissue areas</td>
</tr>
<tr>
<td>Active process</td>
<td>Active process</td>
<td>Passive process</td>
</tr>
<tr>
<td>Morphological features</td>
<td>Sequestration of cytoplasmic material</td>
<td>Swelling of nucleus, organelles and the entire cell</td>
</tr>
<tr>
<td>Blebbing of intact outer membranes</td>
<td>Degradation of ER, Golgi, and partly of mitochondria</td>
<td>Disruption of membranes</td>
</tr>
<tr>
<td>Preserved organelles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromatin condensation</td>
<td>Degradation of ER, Golgi, and partly of mitochondria</td>
<td>Disruption of membranes</td>
</tr>
<tr>
<td>Apoptotic body formation</td>
<td>Formation of autophagosomes</td>
<td>Spilling of cell constituents</td>
</tr>
<tr>
<td>Biochemical features</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate PT</td>
<td>PT?</td>
<td>Severe PT</td>
</tr>
<tr>
<td>Requirement of ATP</td>
<td>Requirement of ATP</td>
<td>ATP-independent</td>
</tr>
<tr>
<td>Mitochondrial efflux of Cyto-C</td>
<td>Other mitochondrial contributions?</td>
<td></td>
</tr>
<tr>
<td>De-polymerization and cleavage of cytoskeleton</td>
<td>Redistribution preservation of the cytoskeleton</td>
<td></td>
</tr>
<tr>
<td>Dependence on caspases and/or AIF</td>
<td>Caspase-independent; role of AIF?</td>
<td></td>
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<tr>
<td>DNA-fragmentation at random?</td>
<td></td>
<td></td>
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<tr>
<td>Non-random DNA fragmentation</td>
<td></td>
<td></td>
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<tr>
<td>Molecular features</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Promoted by Bad, Bim, Bcl, AIF, and mitotic stimuli in absence of survival signals</td>
<td>Promoted by Beclin-1</td>
<td>Prevented by Bcl-2, and PARP-inhibition</td>
</tr>
<tr>
<td>Regulated by p70-S-6 kinase and mTOR</td>
<td>Regulated by p70-S-6 kinase and mTOR</td>
<td></td>
</tr>
<tr>
<td>Inhibited by Bcl-2, Bcl-XL</td>
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</tbody>
</table>
| All three types of cell death exhibit distinct but also similar features at the molecular, biochemical and physiological level as far as known to date. To investigate the type of cell death induced by various triggers, the examination of the morphological features is still a reliable method for an initial discrimination. Many of the morphological features can be determined by middle or high resolution fluorescence phase contrast microscopy in combination with vital staining methods (e.g. Hoechst 33258, propidium iodide, mono-dansylcadaverine) using appropriate fluorescence filters. Some of the morphological criteria need to be examined by electron microscopy, which makes investigations more tedious. To speed up the discrimination analysis phase contrast fluorescence microscopy in combination with biochemical-analytical methods such as monitoring of the stability of cytoskeletal proteins and caspase-mediated cleavage of target proteins by Western blotting, can be used to determine the type of cell death. However, it is not unusual that a cell population undergoes all three death types upon the same stimulus. Even in the same cell the distinct death features may occur. Abbreviations: PT, mitochondrial pore transition; ATP, adenosine triphosphate; Cyto C, cytochrome c; AIF, apoptosis inducing factor; ER, endoplasmatic reticulum; mTOR, mammalian target of rapamycin. ΔΨm, mitochondrial membrane potential.

(type I cell death; see Table 1), in which mitochondria are the relevant organelles which remain preserved throughout the process, whereas actin, cytokeratins and lamins become fragmented [76]. There are reports demonstrating that autophagy proceeds independent of caspases [77,78]. This implicates that several suicide mechanisms evolved in parallel. Recent data, however indicates that cell death, which was triggered by toxins directed against other organelles such as the ER, is also dependent on mitochondrial pore transition [79]. Despite the autophagy-inducing gene beclin1 [80], a Bcl-2-interacting protein, which is homologous to yeast autophagy gene aop6, molecular components of type II cell death still await to be determined. aop genes interact with mTOR (mammalian target of rapamycin) and thus might link mTOR, FK506, and S6-kinase to autophagic processes. Interestingly, type I and type II cell death can occur in mixed form within the same tissue or cell type (see Fig. 1) and even within the same cell. Autophagy which is known
from yeast to the slime mold *D. discoideum* [81] to mammals [76,82] implicates that self-destructing mechanisms already existed before the splitting of ancestral unicellulars into fungi and metazoans. The prime function of autophagy in yeast is the degradation and the recycling of cytoplasmic constituents and organelles in response to nutritional starvation. However, in higher eukaryotic cells its independence of or interdependence with apoptosis needs to be further clarified [83,84]. Experiments that specifically inhibit apoptosis but force or reverse autophagic vacuole formation will elucidate whether autophagic processes are sufficient for cell death induction.

### 5. Cell-death programs as prerequisites of organized life

During embryonic development of the nervous system [85], the finger digits, or the ovary [86], but
also in adult organisms [11], minutely regulated daily death processes maintain health and integrity. Every second, several millions of cells of the human body undergo apoptosis, i.e. in conditions of homeostasis each mitosis is compensated by one event of apoptosis. Such as to select B cell in the germinal centers of lymph nodes by follicular T cells [87], or to remove aged phosphatidylserine-exposing erythrocytes by macrophages—otherwise blood vessels would plug [88,89]. Hence, in the blood system, cell death on program has to be as abundant as cell replication. Once this fine-tuned balance is disturbed (even when occurring at sub-detectable extent), sooner or later life-threatening diseases such as lymphomas and leukemia will become manifest. Lymphocyte selection in germinal centers is based on ligand-receptor mediated cell death [90,91] which is an additional specialization and acquired phylogenetically later to the regulatory repertoire of apoptosis. This type I apoptosis, which is exerted across caspase 8 (extrinsic pathway triggered by, e.g. FasL, TNFα, TRAIL, see Fig. 2), is assumed to be independent of mitochondria [92] and is set in contrast to type II apoptosis (intrinsic pathway triggered by, e.g. toxic compounds, stress, but also during development; see Fig. 2), which is exerted by caspase 9 [93-97]. Interestingly, inhibition of the mitochondrial pathway by Bcl-XL overexpression prevents Fas-triggered apoptosis [98]. This may be explained by the observation that DISC-activated caspase 8 can cross-talk to and activate the mitochondrial caspase 9 by the Bid-bypass [99,100], thereby connecting the extrinsic to the intrinsic pathway and amplifying receptor-mediated death signals (see Fig. 2). Since apoptosis is required for organ development and tissue homeostasis the dis-regulation of cell death programs results in various pathologies. An abnormal resistance to apoptosis induction causes malformations, cancer, or autoimmune diseases due to the persistence of superfluous, mutated cells, or cell-specific immunocytes, respectively. Conversely, acute apoptosis upon infection by toxin-producing microorganisms, during ischemia-reperfusion damage, or infarction will result in enhanced decay of cells, whereas chronically increased apoptosis induces diseases such as neurodegenerative and neuromuscular disorders and AIDS.

6. Apoptosis–necrosis: similar start–different finish

There is convincing evidence that mitochondria are central regulators of apoptosis [101,102]. As one of the very first events in response to multiple stresses or physiological induction, mitochondrial PT causes ion efflux [103] thereby disturbing the mitochondrial membrane potential $\Delta \Psi_m$ [26].

One consequence of $\Delta \Psi_m$ disruption is interference with mitochondrial oxidative phosphorylation resulting in the decrease of ATP [104,105]. Another consequence is the uncoupling of the respiratory chain leading to the hyperproduction of ROS, affection of the plasma redox potential, and subsequent irreversible oxidation of thiols and membrane lipids, as well as the depletion of glutathione [106]. Further, the influx of H$_2$O and Ca$^{2+}$ ions, will cause swelling of the cytoplasm and organelles due to osmotic pressure, and finally the cell bursts and spills the fluids into the pericellular space. This phenomenon describes a classic necrotic phenotype (see Fig. 2). Cytochrome c, which together with Apaf 1 induces caspase 9 in higher eukaryotes, and AIF, which directly translocates into the nucleus to induce caspase-independent stage I apoptosis, are also released through the activated PT–pore complex [16,107] (see Fig. 2). Very likely AIF, which mediates degradation of genomic DNA into 50 kb fragments, exerts its activity during apoptosis and necrosis [108]. Therefore, the extent of $\Delta \Psi_m$ disturbance may determine between life and death as a point of no return.

The PT–pore complex is controlled by the anti-apoptotic molecules Bcl-2 and Bcl-XL [56,109,110] and upon ectopic overexpression, Bcl-2 inhibits PT [56,111] and prevents not only apoptosis [112,113] but also necrosis [114-119]. Thus, early apoptotic onset and necrosis start with similar events such as PT [120-125]. ANT, a component of the PT–pore complex [126], which is also regulated by Bcl-2 [127] exchanges mitochondrial matrix ATP for cellular ADP [121,128]. ATP:ADP ratios are supposed to determine whether a cell will vanish by necrosis or orchestrate an apoptotic funeral [129]. The intensity of noxious stimuli (stress, toxic compounds, or even physiological death factors) correlates with the extent of ATP depletion and therefore, with the type of cell death. Exhausting ATP in consequence of excessive
trauma blocks the apoptotic execution machinery downstream of PT and necrosis prevails [103]. Also specific inhibition of caspases by z-VAD-fmk renders cells to exit by a necrotic phenotype although identical stimuli induce apoptosis when z-VAD-fmk is omitted [52,103,130]. This demonstrates that necrosis–apoptosis decisions are not only taken by ATP. Hypothetically the physiological inhibitors of caspases, the IAP family members, might also determine by which mode cells will demise. This has not been investigated so far. Interestingly, it was shown that caspases can cause PT, thus their activation occurs not only downstream but also upstream of a mitochondrial death decision [131,132]. Nevertheless, the status of $\Delta \psi_m$ which depends on the stress type and impact, not only switches between life and death, but gives also major directives for death modes such as necrosis, type II apoptosis, and caspase-independent apoptosis by AIF (see Fig. 2). The current observations support the notion that a substantial number of effectors of the apoptotic machinery developed within (or from?) the mitochondrial PT–pore complex, which is the primordial necrosis effector, unless barriers that separate and regulate biochemical/metabolic processes (inevitable to maintain “life”) are well preserved.

7. Prevention of necrosis in favor of apoptosis

As demonstrated by various experiments, depletion or replenishment of ATP favors necrosis or apoptosis, respectively [36,43,133,134]. Nevertheless, it is not completely understood, by which ATP-dependent step the apoptosis–necrosis-decision is taken:

1. ATP is required to keep survival pathways such as the AKT pathway active. This however should be expected to decide only between life and death but not apoptosis and necrosis.

2. Although the components for apoptosis are (partly) prefabricated in the cell it has been shown that de novo transcription and translation may be required in some cases. Both processes demand ATP.

3. dATP (and therefore ATP), together with cytochrome c and Apaf 1, is needed for caspase 9 assembly into the active apoptosome [135–137].

4. ATP might just be needed to maintain intact membranes, which are a major feature of apoptosis.

Upon DNA-damage by genotoxic stresses (e.g. radiation, radicals, or alkylating agents), the activation of DNA-repair enzymes and PARP causes the consumption of ATP.

Therefore, in cases of severe DNA-damage the energy level will decrease below certain threshold levels and either energy and/or time are insufficient to undergo the entire repair or apoptosis program and the demise is “necrotic”.

In presence of pro-apoptotic stimuli artificial depletion of ATP by inhibitors of the respiratory chain (e.g. rotenone, [138]), or by interference with glycolysis (deoxyglucose, or S-nitrosoglutathione; [139]), the balance is shifted towards necrosis [117,134]. In contrast, restoration of ATP by enhanced glycolysis (fructose, glucose) restores the apoptotic phenotype [29,43]. This is in agreement with the observation that damages which otherwise would cause necrosis (due to high dosage) result in apoptosis execution when energy-levels are artificially maintained, which has impact for chemotherapy, because unwanted side-effects due to necrotic damage can be avoided [43]. However, acute toxicity might result in accidental cell death so quickly (e.g. primary necrosis) that artificial energy restoration alone would not suffice to proceed with an intrinsic death program.

It is evident that distinct self destruction programs, which stand in apparent contrast to necrosis, co-exist within the same cells and can get started depending on the context and on the stimuli. The fact, that such death-programs evolved despite the high price of energy consumption—necrotic cell death would be “cost free”, evidences that apoptosis and other forms of active cell death are major factors in the evolution of multicellular organisms.

Acknowledgements

We want to thank Prof. W. Bursch for helpful discussions and the donation of mono-dansylcadaverine. This work was supported by the Hezelfeldsche Familienstiftung, the Fund of the Austrian National Bank No. 9298 and the Unruhe Privatstiftung to G.K.
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