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Review

Programmed cell death in protists

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Abstract

Programmed cell death in protists does not seem to make sense at first sight. However, apoptotic markers in unicellular organisms have been observed in all but one of the six/eight major groups of eukaryotes suggesting an ancient evolutionary origin of this regulated process. This review summarizes the available data on apoptotic markers in non-opisthokonts and elucidates potential functions and evolution of programmed cell death. A newly discovered family of caspase-like proteases, the metacaspases, is considered to exert the function of caspases in unicellular organisms. Important results on metacaspases, however, showed that they cannot be always correlated to the measured proteolytic activity during protist cell death. Thus, a major challenge for apoptosis research in a variety of protists remains the identification of the molecular cell death machinery.

Keywords: Protist; Unicellular organism; Programmed cell death; Autophagic cell death; Metacaspase; Evolution

1. Introduction

The existence and evolution of programmed cell death in unicellular organisms is a matter of an ongoing debate. While some researchers claim that programmed cell death is the only explanation for their experimental observations, others hide behind Occam's razor pretending that there is no need for hypotheses on the mode of cell death in protists at all. This review summarizes the current knowledge about cell death of phylogenetically very distant protists. The aim is to show, on the one hand, that there is too much data requiring one or more explanatory model(s), but, on the other hand, that the present data is not sufficient to definitely proof programmed cell death for several of these organisms. Furthermore, I would like to point out hypotheses on the evolution and functions of programmed cell death in protists, although I am aware that it will be difficult to prove or disprove most of these hypotheses experimentally.

In order to get an impression of the diversity of eukaryotic life and to be able to group the protists described below, I suggest to have a look at recent phylogenetic models classifying most eukaryotes into one of six or eight major groups [1–3]. According to these classifications, the opisthokont *Saccharomyces cerevisiae* is phylogenetically quite closely related to animals such as *Caenorhabditis elegans* or other so-called higher eukaryotes having defined programmed cell death machinery. Multicellularity – which was often considered as one of the most important prerequisites for the evolution of programmed cell death – is not restricted to the opisthokonta (metazoa and fungi) and can also be found among amoebozoa (e.g. *Dictyostelium*), heterokonta (e.g. brown algae), and of course plants. To my knowledge – with the exception of the rhizaria including the cercozoa – apoptotic markers (see below) have been observed in unicellular organisms of all six or eight major groups of eukaryotes (Fig. 1); for example: (i) Opisthokonta (*S. cerevisiae* see ref. [4] and this BBA issue for review), (ii) amoebozoa (*Dictyostelium discoideum* [5–7]), (iii) plants (*Dunaliella tertiolecta* [8], *Chlamydomonas reinhardtii* [9], and "*Chlorella*" *saccharophila* [10]), (iv) alveolata (including the apicomplexan parasites *Plasmodium berghei* [11] and *P. falciparum* [12–14], the dinoflagellates [15] *Peridinium gatunense* [16] and *Amphidinium carterae* [17], and the ciliate *Tetrahymena thermophila* [18–25]), (v) heterokonta (*Blastocystis hominis* [26–28]), (vi) discicristata (*Leishmania*

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amazonensis [29,30], *L. donovani* [31–36], *L. major* [37,38], *L. mexicana* [38], *L. infantum* [39–42], *Trypanosoma cruzi* [43–45], and *T. brucei* [46–50]), and (vii) excavata (*Trichomonas vaginalis* [51] and *Tritrichomonas foetus* [52,53]).

2. Apoptotic markers and inducers of cell death in phylogenetically distant protists

Theories and models on programmed cell death are experimentally based on the observation of apoptotic markers such as cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, fragmentation of the nucleus, vesicle formation, externalization of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, oxidative stress, loss of the mitochondrial membrane potential ($\Delta\Psi$), the release of proteins from mitochondria (e.g. cytochrome *c*, endonuclease G, apoptosis-inducing factor), activity of proteases (e.g. caspases), and the cleavage of their substrates.

Morphological markers are usually detected by electron and light microscopy. Chromatin condensation and nuclear shape can be furthermore analyzed using a fluorescent DNA-binding dye such as DAPI. Fluorescent microscopy and flow cytometry are applied to show and quantify the loss of $\Delta\Psi$, binding of labelled annexin V to phosphatidylserine, and fragmentation of DNA (using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling). DNA fragmentation, such as oligonucleosomal laddering, is also analyzed by agarose gel electrophoresis. Labelled peptide substrates and/or suicide inhibitors are used to measure protease activity, and cleaved protein substrates as well as released mitochondrial components can be detected by immunochemical methods. Examples of these methods are given in the references listed in Table 1.

During the last years it became evident that caspase-dependent apoptosis is just one form of programmed cell death using genetically encoded machinery and accordingly the term apoptotic marker may somehow be misleading. Nevertheless, I will use this term in the sense of "marker suggesting a kind of genetically encoded and therefore programmed cell death" without necessarily postulating that the type of cell death can be classified as apoptosis (type I), autophagic cell death (type II), programmed necrosis etc. The occurrence of apoptotic markers and (different) models/classifications of cell death are reviewed for example in refs. [54–58]. Apoptotic markers of the organisms mentioned above are summarized in the following paragraphs and in Table 1.

2.1. Opisthokonta

Although apoptotic markers have been observed earlier in several other unicellular organisms [5,18–20,29,43,46], *S. cerevisiae* (baker's yeast) is certainly the best studied one having a widely accepted programmed cell death machinery that is reviewed in ref. [4] and other articles of this BBA issue. Therefore, I only point out two of many must-read articles on apoptosis in yeast describing (i) the first detection of apoptotic markers including chromatin condensation, DNA

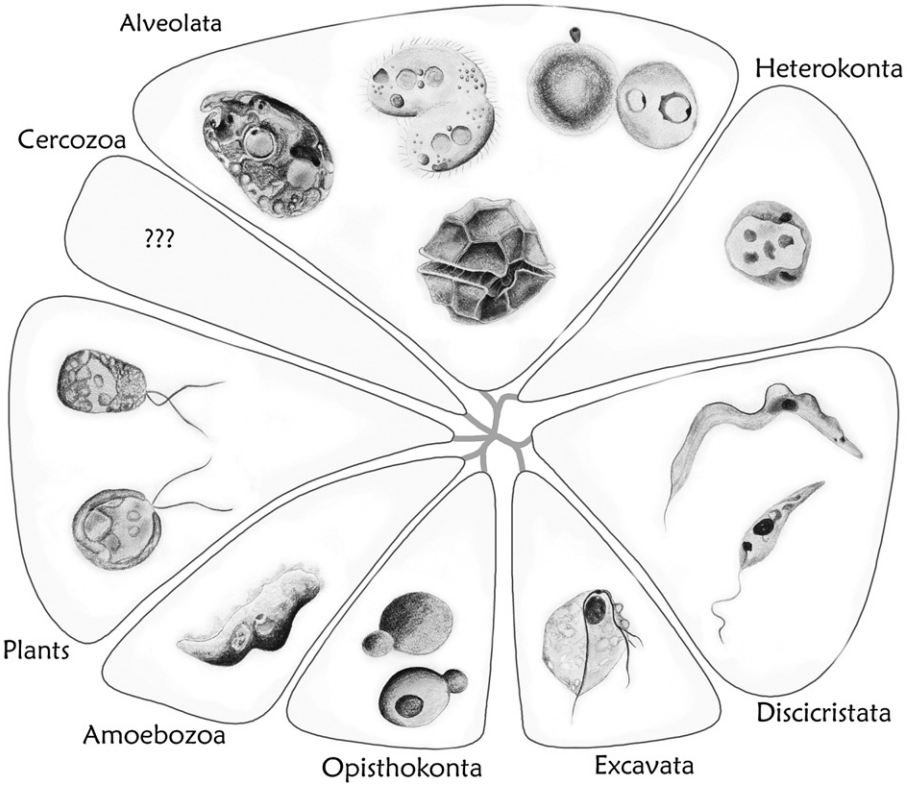

Fig. 1. According to Sandra Baldauf, the vast majority of eukaryotes can be assigned to one of eight major groups [1]. Unicellular organisms of seven of these groups showed apoptotic markers during cell death. For further details see text. Please note that alternative phylogenetic models result in six major groups [2,3].

fragmentation, and externalization of phosphatidylserine in 1997 [59], and (ii) the involvement of the yeast metacaspase in 2002 [60]. These findings reflect the progress from pure phenomenology to deciphering the molecular cell death machinery (e.g. using reverse genetics) and are therefore exemplary for all the following protists. Despite the progress, more exciting discoveries are to be expected from yeast. For example the identification of genes required for autophagy [61] will be very useful for the understanding of different modes of cell death in yeast and other organisms [4,55–58].

2.2. Amoebozoa

Dictyostelium discoideum is a haploid soil living amoeba feeding on bacteria that are found e.g. on decaying leafs. Upon starvation this slime mold forms multicellular aggregates leading within 24 h to a fungus-like structure that contains viable spores and dead stalk cells. Thus, *D. discoideum* is often used as a model organism for differentiation, development, and morphogenesis. The protist can be easily grown *in vitro*, the genome has been sequenced, and the genetic manipulation is well established [62] (http://dictybase.org/). In 1994 Pierre Golstein's group showed that the *in vitro* initiation of stalk cell formation in a mutant strain without morphogenesis led to cell death with an impermeable plasma membrane accompanied by morphological apoptotic markers [5]. The same developmental stimulation of a *D. discoideum* strain carrying a mutation of the autophagy gene *atg1* switched the mode of cell death from a vacuolar, autophagic type to a necrotic type of cell death accompanied by a rapid plasma membrane rupture [63,64]. Further apoptotic markers were detected using amoebae suspension cultures that were either kept in a prolonged stationary phase [6] or that were starved under aggregation-inhibiting conditions with pre-used phosphate buffer as medium [6,7]. In two studies no DNA degradation could be observed [5,6] whereas contradicting results were obtained in a third study [7]. In this work a homolog of human apoptosis-inducing factor (AIF) of *D. discoideum* was shown to be transferred from the mitochondria to the nucleus during cell death, and was suggested to be responsible for the observed DNA degradation [7]. Formation of apoptotic bodies that were engulfed by neighbouring cells (in analogy to the phagocytic systems in mammals and *C. elegans*) was another interesting feature of amoebae cell death [6,7]. In conclusion, *D. discoideum* has become a great non-opisthokont model organism to study different modes of programmed cell death [62].

2.3. Plants

The obligate photoautotroph chlorophyte *Dunaliella tertiolecta* can be cultured in artificial seawater containing inorganic nutrients. When placed in darkness, algae cultures lost their photosynthetic capacity and colour and died after approximately six days. Indeed, *D. tertiolecta* was the first unicellular plant revealing apoptotic markers during cell death [8]. In contrast to *D. tertiolecta*, *Chlamydomonas reinhardtii* is a photoautotroph chlorophyte that is also able to grow in total darkness if acetate is provided as an alternative carbon source. Apoptotic markers in these haploid green algae were observed after UV irradiation of suspension cultures [9]. The third unicellular chlorophyte "*Chlorella*" *saccharophila* was isolated from a glacier and killed by heat stress which also resulted in morphological and molecular apoptotic markers [10]. Among these algae, *C. reinhardtii* – which is sometimes called "green yeast" – is the best established model organism. Furthermore, its genome has been recently sequenced (http://www.chlamy.org/) and it might therefore be highly suited to study cell death in plants in general.

2.4. Alveolata

Alveolates (dinoflagellates, apicomplexa, and ciliates) have systems of cortical alveoli (vesicles) of mostly unknown function directly beneath their plasma membranes. Furthermore, they often contain a plastid-like organelle probably acquired by secondary endosymbiosis (a eukaryote already containing a primary plastid was engulfed by another host eukaryote and reduced to an organelle) [1–3].

(i) Most dinoflagellates are marine and are not commonly used for research despite their ecological relevance. These flagellated protists are often photoautotroph – forming a major part of the "large" phytoplankton – but can be also mixotrophic, heterotrophic, micro-predators, symbionts or even parasites [2,3,65]. The first apoptotic markers in a dinoflagellate were observed in *Peridinium gatunense* in 1999: Blooms of this photosynthetic fresh water organism in Lake Kinneret temporarily led to very high cell densities but were sharply terminated in summer when the local pH rose and the concentration of dissolved CO_2 became limited. Cell death in summer was accompanied by oxidative stress probably due to the formation of H_2O_2 during photosynthesis (using O_2 as an alternative electron acceptor at low CO_2 concentrations). Plasma membrane permeabilization only occurred at a late stage of cell death, and removal of H_2O_2 by exogenous catalase inhibited cell death [16]. Another ecologically important dinoflagellate-dependent cell death event is coral bleaching: A morphological study revealed that both the symbiotic dinoflagellate and its host (the sea anemone *Aiptasia*) showed markers for apoptosis-like and necrosis-like cell death upon heat stress [15]. In analogy to darkness-induced cell death in the chlorophyte *D. tertiolecta*, the marine photosynthetic dinoflagellate *Amphidinium carterae* also died upon light-deprivation and culture senescence. However, the type of cell death of *A. carterae* was clearly different from *D. tertiolecta* and shared more similarities with cell death of *D. discoideum* [17]. Further information on programmed cell death in dinoflagellates can be found in a nice review by Maria Segovia [65].

(ii) Apicomplexans are very successful specialized parasites having unique apical organelles called rhoptries and micronemes that are involved in cell invasion. *Plasmodium* spp. cause malaria and replicate asexually in the mosquito (sporogony) and the vertebrate host (schizogony). Apart from the intraerythrocytic asexual blood stages, "female"

Table 1
Apoptotic markers in unicellular organisms

Organism	Conditions/inducer	Apoptotic markers
Saccharomyces cerevisiae	Various [4,59,60]	DNA fragmentation; chromatin condensation; vesicle formation; membrane blebbing; externalization of phosphatidylserine; loss of $\Delta\Psi$; oxidative stress; release of mitochondrial proteins; increased caspase-like activity [4,59,60]
Dictyostelium discoideum	Starvation of amoebae in the presence of cAMP followed by initiated differentiation to stalk cells [5,7]; starvation of aggregation-inhibited amoebae [6,7] and prolonged stationary cultures [6]	Chromatin condensation [5,7]; late plasma membrane permeabilization [5,7]; vacuolization and vesicle formation [5–7]; cytoplasmic condensation/cell shrinkage [5–7]; externalization of phosphatidylserine [6,7]; loss of $\Delta\Psi$ [6,7]; DNA fragmentation [7]
Dunaliella tertiolecta	Darkness/light-deprivation [8]	DNA fragmentation; chromatin condensation; loss of nuclei; increased caspase-like activity [8]
Chlamydomonas reinhardtii	UV irradiation [9]	DNA fragmentation and laddering; cell shrinkage; vacuolization; nuclear fragmentation; externalization of phosphatidylserine [9]
"Chlorella" saccharophila	Heat stress [10]	DNA fragmentation; chromatin condensation; cell shrinkage; increased caspase-like activity [10]
Plasmodium berghei	Development of parasite midgut stages (ookinetes) in the mosquito [11]	DNA fragmentation; chromatin condensation; externalization of phosphatidylserine; caspase-like activity [11]
Plasmodium falciparum	Treatment of blood stages with antimalarial drugs (chloroquine) [12–14], H_2O_2 [13], etoposide [14]	DNA fragmentation [12–14]; loss of $\Delta\Psi$ [14]; morphological markers ("crisis forms" [12–14]) suggesting nuclear fragmentation, membrane blebbing and vesicle formation [13]
Symbiodinium spp.	Heat stress [15]	Chromatin condensation; intact plasma membrane; vacuolization and vesicle formation; cytoplasmic condensation [15]
Peridinium gatunense	Decrease of dissolved CO_2; H_2O_2 [16]	DNA fragmentation, cell shrinkage; increase in reactive oxygen species; late plasma membrane permeabilization [16]
Amphidinium carterae	Darkness/light-deprivation [17]	Cell shrinkage; vacuolization [17]
Tetrahymena thermophila	(Starvation induced) conjugation [18–20,22–25]; staurosporine [21]	DNA fragmentation [18–20,23,25]; chromatin and nucleus condensation [18–25]; nuclear resorption [18,22,23]; membrane blebbing [21]; vesicle formation [21]; caspase-like activity [23,24]
Blastocystis hominis	Growth in soft agar [26]; addition of a monoclonal antibody against a surface glycoprotein in suspension cultures [27]; antibiotic (metronidazole) [28]	DNA fragmentation [27,28]; cell shrinkage [27,28]; chromatin condensation [26,27]; nuclear fragmentation [26]; vesicle formation [26–28]; membrane blebbing [27,28]; externalization of phosphatidylserine [27,28]
Leishmania amazonensis	Heat shock of promastigotes in the presence of Ca^{2+} [29]; treatment of amastigotes with nitric oxide derived from NO-donating compounds or activated macrophages [30]	DNA fragmentation [29,30]; chromatin condensation [29]; nuclear fragmentation [29]; vesicle formation [29]; cell shrinkage [30]
Leishmania donovani	Treatment of promastigotes with H_2O_2 [31,33], amphotericin B [32], Hoechst 33342 [34], camptothecin [35,36], or antimicrobial peptides [79]; promastigote stationary phase culture [32]	DNA fragmentation [31–35]; chromatin condensation [31–33,35]; cell shrinkage [31]; nuclear fragmentation [31–33]; vesicle formation [35,79]; membrane blebbing [35,79]; decrease in glutathione [31,33,35]; increase in reactive oxygen species [33,35,36]; externalization of phosphatidylserine [35]; caspase-like activity [31,32,35,36]; loss of $\Delta\Psi$ [32,33,35,36]; release of cytochrome *c* [35]
Leishmania major	Treatment of promastigotes with staurosporine [37]; serum removal [38]; heat shock [38]; treatment of amastigotes with nitric oxide derived from activated macrophages [38]	DNA fragmentation [37,38]; chromatin condensation [37,38]; cell shrinkage [37]; nuclear fragmentation [37,38]; vesicle formation [37,38]; externalization of phosphatidylserine [37]; caspase-like activity [37,38]; loss of $\Delta\Psi$ [37]; release of cytochrome *c* [37]
Leishmania infantum	Treatment of cell free amastigotes with Sb^{3+} [39]; treatment of promastigotes with trans-platinum complexes [40]; heat shock [41,42]	DNA fragmentation [39,41]; externalization of phosphatidylserine [40,41]; loss of $\Delta\Psi$ [41,42]; increase in reactive oxygen species [42]
Trypanosoma cruzi	Treatment of epimastigotes by heat stress [43], starvation [43], low cell density [43], or human serum [43–45]	DNA fragmentation [43–45] ; caspase-like activity [44]; increase in reactive oxygen species and decrease in low molecular weight antioxidants [45]; externalization of phosphatidylserine [45]; slight decrease of $\Delta\Psi$ [45]; release of cytochrome *c* [45]
Trypanosoma brucei	Treatment of procyclic forms with the lectin concanavalin A [46] or reactive oxygen species [47]; high density cultures [49] or treatment of bloodstream forms with prostaglandin D_2 [48,50]	DNA fragmentation [46–50]; chromatin condensation [46,48,50]; vesicle formation [46,48,50]; externalization of phosphatidylserine [48–50]; decrease of $\Delta\Psi$ [48,50]; increase in reactive oxygen species [50]
Trichomonas vaginalis	Treatment of axenic suspension cultures with staurosporine, etoposide, doxorubicin, or metronidazole [51]	DNA fragmentation; chromatin condensation; nuclear fragmentation; vesicle formation; externalization of phosphatidylserine; vacuolization [51]
Tritrichomonas foetus	Treatment of suspension cultures with H_2O_2 [52] or the fungicide griseofulvin [53]	Chromatin condensation [52]; nuclear fragmentation [52,53]; membrane blebbing [52,53]; vacuolization [52,53]; caspase-like activity [52]; externalization of phosphatidylserine [53]

and "male" gametocytes enter the mosquito midgut during an infected blood meal. Gametes are rapidly formed and fuse to a zygote differentiating into motile ookinetes that invade the mosquito by traversing the midgut epithelium. Using the rodent model parasite *P. berghei*, Hilary Hurd's group discovered in 2002 that about half of the ookinetes showed molecular and morphological apoptotic markers *in vitro* and in *Anopheles stephensi* [11]. In addition, apoptotic markers were observed in the human parasite *P. falciparum* e.g. after treatment of blood stage cultures with the antimalarial drug chloroquine [12–14]. However, it is quite difficult to analyze apoptotic markers in *Plasmodium* parasites [13], and several groups state that very few or none of these markers could be detected during *Plasmodium* cell death at all [66–68]. Nevertheless, *Plasmodium* spp. are interesting model organisms to study the mode of cell death for example because of their medical relevance and the available genetic tools and genome databases (http://www.plasmodb.org/plasmo/home.jsp). For further review see also refs. [13,69,70].

(iii) Ciliates, such as *Tetrahymena thermophila* are binuclear organisms with two distinct types of nuclei: Polyploid somatic macronuclei and diploid germline micronuclei. Accordingly, *T. thermophila* became a well known model organism for so-called programmed nuclear death of the parental macronucleus during a sexual process termed conjugation [71]. Please note that both conjugating cells survive and therefore programmed nuclear death is not a mode of cell death (however, in one study an autophagic kind of cell death without detectable DNA fragmentation was induced after treatment of *T. thermophila* suspension cultures with staurosporine [21]). In 1992 David Allis' group discovered that the removal of the parental macronucleus and its DNA was not only coupled to highly regulated morphological changes resembling apoptosis but was also genetically encoded [18]. The same group identified the "programmed DNA degradation protein 1" (Pdd1p) which might be a molecular link between chromatin condensation and DNA degradation in late stage parental macronuclei [20]. Initiation of DNA degradation resulting in high molecular weight fragments seemed to be necessary for chromatin condensation and nuclear death [19]. Furthermore, degradation of the condensed macronucleus was shown to occur in an acidic phosphatase-containing autophagosome [22]. No general loss of mitochondrial membrane potential was observed during nuclear death, but surprisingly the activated marker dye was found in the degenerating macronucleus suggesting a colocalization of the nucleus and permeabilized mitochondria in an autophagosome [23,25]. Indeed, endonuclease G-like activity from isolated *Tetrahymena* mitochondria could be identified, pointing to an interesting possibility how mitochondrial proteins might selectively trigger DNA fragmentation and removal of the macronucleus [25]. In summary, *T. thermophila* is highly suited to selectively investigate nuclear apoptotic markers. The linty predator will be even more valuable in the future because it can be easily cultured and genetically manipulated, and the macronuclear genome has been recently sequenced (http://www.ciliate.org/). Further details on nuclear death and apoptosis-like phenomena in ciliates are reviewed in ref. [71].

2.5. Heterokonta

Blastocystis hominis is a highly abundant parasite being transmitted as a cyst via the fecal-oral route. It infects the gastrointestinal tract of humans and animals and adopts a variety of different morphologies depending on the environment. Several cells in soft agar colonies of *B. hominis* cultures showed suspicious morphology (without further induction of cell death) [26]. In addition, cell death in suspension cultures was induced in the presence of a monoclonal antibody against a surface glycoprotein, whereas the presence of a control antibody did not cause an increase of cell death accompanied by apoptotic markers [27]. A third work by Kevin Tan's group revealed similar results after inducing cell death with the antibiotic metronidazole [28]. In the latter cases the central vacuole seemed to play a major role in removing numerous vesicles during cell death, and even though no DNA-laddering was observed, DNA fragmentation could be detected in a TUNEL-assay [27,28]. To my limited knowledge, the genome has not been sequenced yet and genetic tools for *Blastocystis* are unfortunately not well established. The future will tell whether this parasite has the potential to become a suited model organism. For further review on cell death in *B. hominis* see also refs. [72,73].

2.6. Discicristata

Cell death of different developmental stages of kinetoplastida of the genus *Trypanosoma* and *Leishmania* was shown to be coupled to the occurrence of apoptotic markers (for extensive review, see also refs. [74–78]). These flagellated parasites all have a kinetoplast (a DNA-containing body located within the single mitochondrion) and cause either different forms of leishmaniasis or trypanosomiasis such as Chagas disease (South American *T. cruzi*) and sleeping sickness (African *T. brucei*). The kinetoplastida mentioned here are all transferred between an insect vector and mammals. The story on programmed cell death in trypanosomes began when Jean-Claude Ameisen et al. discovered apoptotic markers in *T. cruzi* during the *in vitro* proliferation and differentiation of *epimastigotes* in 1995 (the epimastigote form multiplies *in vivo* in the insect gut, becoming non-dividing infective metacyclic trypomastigotes) [43]. Induction of *T. cruzi* cell death and DNA fragmentation by human serum could be inhibited by L-arginine-dependent synthesis of nitric oxide [44]. In contrast to nitric oxide preventing cell death *in vitro*, mitochondrial super oxide anions caused oxidative stress and promoted serum-induced epimastigote death. Accordingly, cell death was inhibited by overexpression of mitochondrial super oxide dismutase in *T. cruzi* [45]. Reactive oxygen species – generated *in vitro* using xanthine oxidase – also induced cell death of *procyclic* forms of *T. brucei* (procyclic forms of this parasite are found *in vivo* in the gut of the Tsetse fly). This pathway was suggested to be Ca^{2+}-dependent because an increase

in cytosolic and nuclear Ca^{2+} occurred before DNA fragmentation (which furthermore could be inhibited by chelators) [47]. Another way to induce cell death in suspension cultures of procyclic *T. brucei* is the addition of concanavalin A, a glucose- and mannose-specific lectin binding to glycoproteins [46]. Cell death in *bloodstream* forms of *T. brucei* was induced by adding prostaglandin D_2 [48] or its derivatives [50] to the medium and was accompanied by a large set of apoptotic markers [48,50] including reactive oxygen species [50]. Addition of low molecular weight antioxidants [50] or expression of alternative oxidase [49] inhibited cell death of *T. brucei*.

The first apoptotic markers in the parasite *Leishmania amazonensis* were observed after incubation of *promastigote* forms in the presence of additional Ca^{2+} at 34 or 37 °C (motile flagellated promastigotes are found *in vivo* in the insect gut and are usually cultured below 30 °C). In the absence of Ca^{2+} no ultrastructural apoptotic markers were observed [29]. Promastigotes of the causative agent of often fatal Kala-azar, *L. donovani*, showed apoptotic markers after aging [32] or treatment with H_2O_2 [31], the antileishmanial drug amphotericin B [32], the DNA-binding dye Hoechst 33342 [34], or camptothecin [35,36]. H_2O_2- and camptothecin-induced death of *L. donovani* promastigotes was furthermore shown to be linked to an increase of cytosolic Ca^{2+} and could be inhibited by low molecular weight antioxidants [33,36]. In contrast to these studies, no DNA fragmentation but a kind of autophagic type of cell death was observed when *L. donovani* promastigotes were treated with antimicrobial peptides [79]. Promastigotes of other *Leishmania* species, *L. major* and *L. infantum*, were shown to be killed after incubation with the broad protein kinase inhibitor staurosporine [37] or *trans*-platinum complexes [40], respectively. In addition, heat stress-induced cell death of *L. infantum* did not seem to depend on caspase-like activity but could be mitochondrially triggered since expression of mammalian Bcl-X_L inhibited cell death [41] which was coupled to oxidative stress [42]. In the mammalian host, *Leishmania* parasites infect macrophages and differentiate into intracellular *amastigote* forms: Cell death accompanied by DNA fragmentation in non-flagellated *L. amazonensis* and *L. major* amastigotes could be induced by nitric oxide [30,38] and was suggested to be proteasome-dependent [30]. The nuclease executing DNA fragmentation in *L. major* amastigotes was suggested to be a large (45–49 kDa), endogenous, Ca^{2+}- and Mg^{2+}-independent, site-unspecific endonuclease [38]. DNA fragmentation during cell death of *L. infantum* amastigotes could be furthermore induced by Sb^{3+} which is interesting from a medical perspective since antimonials are used as antileishmanial drugs [39]. In summary, there is a lot of data supporting the view that programmed cell death can be also induced in these ancient parasites. Furthermore, several of the studies highlight the relevance of Ca^{2+} and oxidative stress during programmed cell death suggesting that different inducers trigger similar pathways in kinetoplastida. The existence of genetic tools and several sequenced kinetoplastida genomes (http://www.sanger.ac.uk/Projects/Protozoa/, http://www.tigr.org/parasiteProjects.shtml) will probably help deciphering the genes and proteins that are required for the observed phenomena.

2.7. Excavata

The trichomonads *Trichomonas vaginalis* and *Tritrichomonas foetus* are fascinating parasites to study the mode of cell death since both organisms possess hydrogenosomes instead of mitochondria. Even though these double-membrane-bounded organelles have a common ancestor – sharing similar protein import components and synthesizing iron–sulfur clusters – hydrogenosomes lack many components of classical mitochondria and generate for example molecular hydrogen instead of consuming oxygen [80]. It is therefore interesting to study (i) whether hydrogenosomes are involved in cell death at all and (ii) whether there are evolutionary conserved non-mitochondrial programmed cell death pathways in trichomonads [81,82]. So far, several apoptotic markers have been observed in these organisms supporting the hypothesis that for example a kind of autophagic type of programmed cell death exists [51–53]. Nevertheless, the field is very young and more studies are required to understand or exclude a putative role of hydrogenosomes during cell death.

3. Metacaspases and proteolytic activity during cell death

Most of the classical genes encoding apoptosis in metazoa are missing in protists, but there are a few genes that are suggested (or even shown) to play a role in programmed cell death in unicellular organisms. Apart from single examples such as AIF in *Dictyostelium* [7], Pdd1 in *T. thermophila* [20], and Sir2 in *Leishmania* [83], the most promising candidates encode metacaspases. These proteases were identified *in silico* in unicellular organisms and plants [84] and belong to the C14 family of clan CD enzymes possessing a conserved caspase-like catalytic dyad (composed of a histidine and a cysteine residue) [85]. In contrast to caspases – which are specific for aspartate as the C-terminal amino acid (P1) of a tetrapeptide cleavage motif [86] – metacaspases were shown to be specific for basic amino acid residues [87,88] (see also ref. [70] for review and further considerations). Accordingly, all results obtained from either cell death inhibition studies or caspase-activity measurements in protists using aspartate-containing compounds have to be reinterpreted and cannot reflect a *direct* effect of these compounds on metacaspase activity! In addition, it has been recently suggested that metacaspases could have functions independent of programmed cell death or that in several cell death scenarios metacaspase/paracaspase gene disruption does not influence cell death at all [67,89–92].

Nevertheless, there is a lot of data available showing (changes in) caspase-like activity during cell death in protists, and therefore it cannot be ruled out that other proteases act independently or up- or downstream of a metacaspase: For example cell death of *P. berghei* midgut stages could be prevented by the peptide-derived suicide inhibitors zVAD-fmk and zDEVD-fmk, but not by zYVAD-cmk [11]. In darkness, an increase in caspase-like activity was furthermore observed for *D. tertiolecta* [8]. Cleavage of DEVD-pNA was also increased in cell extracts of heat-shocked "*C.*" *saccharophila* cultures and addition of ac-DEVD-CHO inhibited cell death [10]. Caspase-

like activity was even detected in cytoplasmic vesicles of *T. thermophila* when a condensed parental macronucleus was present. Addition of zVAD-fmk or ac-YVAD-CHO significantly blocked macronuclear chromatin condensation and elimination. However, no colocalization of the macronucleus and the vesicular caspase-like activity was observed, suggesting that the enzymatic activity did not play a direct role in the degradation of the parental macronucleus [24]. Another study on *T. thermophila* cell extracts also revealed a slight time- and substrate-dependency of caspase-like activity during conjugation (using ac-IETD-pNA and ac-LEHD-pNA as substrates) [23]. In *P. gatunense*, addition of the cysteine protease inhibitor E64 abolished cell death but led to cyst formation [16]. Some controversial findings – suggesting either caspase-like, calpain-like or proteasome-like activity during cell death – were also made in kinetoplastids and might be due to different death inducers, species or life stages: For example, cell death in *T. cruzi* epimastigotes was prevented by ac-DEVD-CHO [44], whereas serine and cysteine protease inhibitors (such as zDEVD-smk, zVAD-fmk, calpain I inhibitor, tosyl-lysylchloromethane, PMSF, and E64) were without effect on Ca^{2+}-dependent cell death in procyclic *T. brucei* [47]. Addition of zVAD or DEVD-CHO did not rescue *T. brucei* bloodstream forms as well [48], and NO-induced cell death of *L. amazonensis* amastigotes could not be inhibited by zDEVD-cmk, zVAD-fmk or E64, whereas proteasome inhibitors had a time-dependent effect [30]. In contrast, H_2O_2- or amphotericin B-induced cell death of *L. donovani* promastigotes led to an increase of caspase-like activity that could be (partially) inhibited using zDEVD-fmk [31,32] but not by calpain or cathepsin inhibitors [32]. E64 and caspase-inhibitor BAF both prevented DNA fragmentation (but not cell death!) in promastigotes of *L. major* after treatment with staurosporine, whereas the proteasome inhibitor lactacystin had no effect [37]. These data are of course confusing and the reader is probably happy to turn to *Dictyostelium* because of the lack of evidence for any (para)caspase-like activity during amoebae cell death [7,91,92] although several conditions and inhibitors were tested (including zVAD-fmk, ac-DEVD-CHO, BOC-Asp-fmk, DEVD-fmk, YVAD-fmk, leupeptin, E64, or FA-fmk).

In summary, there is no clear picture of the proteases that were active in these different experiments and the substrate specificity of metacaspases shows that one has to be careful with the interpretation of proteolytic activity. Much more work is required to understand the execution of cell death in protists on a molecular level. Apart from generating metacaspase knock-out and overexpressing strains from different eukaryotes, it will be certainly helpful to use (metacaspase-specific?) arginine- or lysine-containing peptides as substrates and inhibitors instead of the classical caspase substrates.

4. Physiological functions and evolution of programmed cell death

Depending on the organism and its life style, there are several hypotheses on the origin and functions of programmed cell death in protists. The physiological function of autophagic cell death in *D. discoideum* seems to be the stalk formation [5] in analogy to developmental cell death in metazoans. Results on the chlorophyte *C. reinhardtii* suggest that UV-irradiated cells render their culture medium (the environment) more protective toward UV-mediated killing of freshly grown cells. Thus, dying cells might confer protection to bystander cells against UV irradiation [9]. However, it is also possible that programmed cell death in chlorophytes confers no ecological or evolutionary fitness at all. It was therefore suggested that bacterial and viral genes became incorporated into marine heterotrophs and autotrophs and were laterally transferred via further viral infections earlier in evolution. Thus, the observed cell death was suggested to be the relic of what is now the common response to biotic and abiotic stress for higher plants [8,93,94]. In the case of the blooming dinoflagellates, Vardi et al. suggested that by allowing only the best adapted individuals to establish cysts, while eliminating less healthy members of the community at the end of the bloom, programmed cell death might confer a selective advantage to a population during subsequent seasons [16]. In the case of symbiotic dinoflagellates a programmed cell death might remove damaged cells that generate oxidative stress [15] or that are virally infected [65] thereby protecting the host and the surrounding protist population. Programmed cell death of parasites such as *P. berghei* or *L. donovani* insect gut stages was suggested to play a major role in limiting parasite infection intensity, thus avoiding the death of the host [11,31]. Similar scenarios might be also possible for other life stages and parasites (see for example refs. [13,69,76,77] for review). What might be the physiological triggers of programmed cell death in protists? For example, death of insect stages of *L. amazonensis* [29] or of non-infective *T. cruzi* epimastigotes [43] at 37 °C could reflect a physiological condition upon infection. The advantage of a programmed cell death might be the avoidance of the liberation of harmful compounds and a decreased immune response of the mammalian host. Apart from changes in temperature, chemical compounds might trigger cell death of individuals of a protist subpopulation. Short-lived prostaglandin D2 is the major prostanoid produced and secreted by a non-dividing bloodstream form of *T. brucei*. Indeed, this compound induces cell death and could therefore be involved in the regulation of parasitemia removing the non-dividing form of the parasite population [48,50,77]. Since several protist populations are thought to be clonal, programmed cell death might also be an altruistic mechanism to promote and maintain genetic stability.

5. Conclusion

Having summarized the available data, I once again got the impression that programmed cell death exists in several protists, although a detailed description of the molecular machineries executing cell death is still missing. The identification of genes encoding such cell death machinery should be the major goal during the next years in order to experimentally convince the sceptics. This might also solve the question whether programmed cell death originates from gene transfers that occurred several times during evolution or has deep evolutionary roots e.g. before the six/eight major groups of eukaryotes diverged.

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