Trypanosomatid apoptosis
‘Apoptosis’ without the canonical regulators

Despina Smirlis* and Ketty Soteriadou
Laboratory of Molecular Parasitology; Department of Microbiology; Hellenic Pasteur Institute; Athens, Greece

Apoptosis is a regulated process of cell death originally described in multicellular organisms contributing to their development and functionality. There is now increasing experimental evidence that a similar form of cell death is operative in unicellular eukaryotes, including trypanosomatids of the genera Trypanosoma and Leishmania. The determination of ancestral executors and regulators of ‘apoptosis’ in these protozoa belonging to the most primitive eukaryotes that appeared on Earth 1.5 billion years ago, provide an exciting challenge in the understanding of the evolution of apoptosis-regulating processes. A review of the present knowledge of trypanosomatid apoptosis points to the fact that these dying protozoa acquire common apoptotic morphological features as metazoan cells, although they lack many of the molecules accepted today as canonical apoptosis mediators (Bcl-2 family members, caspases, TNF-related family of receptors). Herein, we discuss how the knowledge of regulators and executors of trypanosomatid apoptosis may provide answers to the gaps concerning the origin of apoptosis. The aim of this addendum is to emphasize the need for classifying the ancestral death program and to discuss how this relates to the complex death programs in multicellular lineages, with the hope to stimulate further enquiry and research into this area.

Apoptosis is a form of cell death in which a programmed sequence of events leads to the elimination of cells without inducing or evoking inflammatory responses. Although it was originally considered a characteristic of multicellular organisms, recent data suggest that a mechanism with many similarities to metazoan apoptosis is operative in unicellular eukaryotes, including trypanosomatids of the genera Trypanosoma (T. cruzi and T. brucei) and Leishmania. Trypanosomatids are a group of kinetoplastid protozoa, and are considered as direct descendants of the first eukaryotes that appeared on Earth 1.5 billion years ago, occupying a pivotal position between the ancestral kingdom of Bacteria and the four derived eukaryotic kingdoms. These parasites that display complex life cycles, with multiple differentiation forms alternating between mammalian and insect hosts, are the causative agents of diseases such as Chagas disease (American trypanosomiasis), African sleeping sickness (African trypanosomiasis) and Kala-azar (visceral leishmaniasis). It is estimated that more than 20 million people are infected and suffer extensively by these protozoan parasites and more than 100,000 die from these parasitic diseases per year.

Induction of ‘apoptosis’ in these parasitic protozoa, is suggested to be useful in the establishment of infection in several ways, including the control of parasite numbers. The control of parasite numbers in response to limited resources, within the insect vector or mammalian host, would provide a means for the perpetuation of infection. For example, it has been demonstrated that in the insect gut of the tsetse fly the numbers T. brucei parasites remain constant after an infected blood meal due to the death of a significant...
number of cells displaying a characteristic apoptotic morphology.\textsuperscript{5,6} Additionally, a system of self-regulation of the numbers of bloodstream form trypanosomes in the mammalian host has also been proposed, as these parasites produce prostaglandin D\textsubscript{2}, which induces their own cell death.\textsuperscript{7} Moreover this form of cell death is advantageous for the modulation of the host immunity required for the establishment of infection.\textsuperscript{8} For example it has been suggested that a regulated cell death may be useful to trypanosomatids for the avoidance of an inflammatory response and for the silencing of the immune system. Thereby apoptosis of \textit{Leishmania} spp. mediates the silencing of human phagocytes by inducing TGF-\(\beta\) production, allowing in this way the intracellular survival of non-apoptotic parasites.\textsuperscript{9} Apart from the modulation of parasite densities and the immune system, this form of cell death may be used by trypanosomatids to optimize their biological fitness.\textsuperscript{10} That is, the coupling of cell-cycle control and proliferation with this regulated form of cell death,\textsuperscript{2} provides a means for the selection of the fittest cells in hostile environments. Therefore a program of self-destruction in these protozoa, would provide an evolutionary advantage in regulating complex interactions between unicellular and multicellular organisms and the persistence of stable host/parasite interactions for the establishment of infection. A better knowledge of this self-destruction program in these protozoa will not only contribute into gaining insights on the molecular events that play a role in the establishment of infection, but will also provide answers to when and why these unicellular eukaryotes die in a regulated way.\textsuperscript{11-13} Furthermore, the detailed knowledge of how these protozoa die will provide a better understanding of the origin of eukaryotic apoptosis, and of the basic components of the apoptotic machinery.

This form of controlled cell death in trypanosomatid parasites is characterized by common morphological features to apoptotic multicellular lineages as described by the Nomenclature Committee on Cell Death (NCCD).\textsuperscript{14} These include cell rounding up, reduction of the cellular volume, membrane blebbing, chromatin condensation and engulfment by host phagocytes.\textsuperscript{15} In addition to these morphological features, the biochemical outcomes of this type of cell death as described for metazoan cells includes the dissipation of the biochemical membrane potential (\(\Delta\psi\text{m}\)), phosphatidylserine (PS) exposure to the outer leaflet of the plasma membrane, cytochrome c release and maintenance of an intact plasma membrane until late stages of the process.\textsuperscript{1,2} However, although these parasites have apoptotic phenotypes similar to metazoans, the main players of apoptosis such as caspases, Bcl-2 family members, TNF-related family of receptors and the caspase-activated DNAses are not present in these ancient eukaryotes.\textsuperscript{16} We will use the term 'apoptosis' here, for an induced cell death that shows considerable number of apoptosis hallmarks according to the NCCD\textsuperscript{14} but executed by non-canonical regulators.

Despite the lack of many proteins known to participate in metazoan apoptosis; executors of this form of cell death in trypanosomatids are slowly coming into light, and have been recently revised.\textsuperscript{2} Presently, there is increasing evidence that metacaspases a group of proteases with similar folds but different enzymatic activity to caspasas, may be involved in 'apoptosis'.\textsuperscript{17-19} The caspase-like activity however, observed by many investigators in dying trypanosomatids, has been associated with the activation of lysosomal cathepsin-like proteases.\textsuperscript{20} Besides the pre-mentioned activation of proteases, nucleases that degrade DNA participate in the dismantling of these protozoan cells. Such an example is EndoG, a well characterized nuclease from multicellular lineages implicated in caspase-independent programmed cell death by its translocation from the mitochondrion to the nucleus.\textsuperscript{20} In a similar way, trypanosomatid EndoG acts as a pro-apoptotic nuclease,\textsuperscript{21,22} present with TatD-like nuclease (a prokaryotic nuclease) and FEN-1 (a DNA repair protein) in two separate protein complexes.\textsuperscript{23} These complexes are formed upon the translocation of EndoG to the nucleus from the mitochondrion of dying \textit{L. donovani} promastigotes.\textsuperscript{23}

Consequently, mitochondrial dysfunction in these protozoan parasites plays an important role in the apoptotic effector phase, as in metazoan cells.\textsuperscript{24,25} Upon different triggers of cell death in trypanosomatids, there is an interplay between reactive oxygen species (ROS) generation, Ca\textsuperscript{2+} overload and mitochondrial dysfunction, indicated by changes in mitochondrial membrane potential (\(\Delta\psi\text{m}\)) and release of cytochrome c.\textsuperscript{24,26-28} Although cytochrome c release may not amplify trypanosomatid 'apoptosis' by caspase activation, it has been suggested that such a mechanism may be important for the disruption of the electron transport leading to the generation of lethal levels of ROS.\textsuperscript{29,30} Notably, in \textit{Leishmania} spp. the inhibition of the mitochondrial respiratory chain results in ROS generation and finally in an apoptotic-like cell death,\textsuperscript{30} suggesting that the signal initiating the death process in these organisms originates from the mitochondrion. Recently Zalila et al. showed that in \textit{L. major} oxidative stress induced the metacaspase processing into an active form, directly linking ROS with the cell death pathway in this species.\textsuperscript{31} Moreover, elevation of ROS, is associated with the deregulation of Ca\textsuperscript{2+} homeostasis and both these factors participate to amplify trypanosomatid 'apoptosis', mediated by mitochondrial dysfunction.\textsuperscript{24,26-28}

As the mitochondrial function appears to be a decisive mechanism of cell death in these organisms, it may have major implications for the phylogeny of apoptosis. For example, it is widely accepted that eukaryotic cells are descendants of anaerobic organisms that survived in an oxygen-rich world by engulfing aerobic bacteria, in a symbiotic relationship. The current knowledge of trypanosomatid 'apoptosis' supports the hypothesis by Blackstone and Green who suggested that the evolutionary origins of apoptosis can be traced back in time to the pro-mitochondrial electron transport chain and ROS, assumed to be the central mediators of signaling between the mitochondrion symbiont and the host cell.\textsuperscript{31} An emerging scenario for the origin of the eukaryotic apoptotic system involves the acquisition of several central apoptotic mediators from the mitochondrion, as a consequence of endosymbiosis.\textsuperscript{32} Indeed many domains of apoptosis-associated proteins present in phylogenetically diverse unicellular eukaryotes,\textsuperscript{33} are also found in bacteria.\textsuperscript{33} Such a mechanism
could enable endosymbionts to kill their host cells in unhospitable environments (i.e. starvation) and efficiently use the corpse of the assassinated host before they moved to another host.\textsuperscript{14}

Although this hypothesis may provide some explanation on the evolution of apoptosis in multicellular lineages, the terminology and the classification of this form of cell death has been a matter of ongoing debate, which needs to be addressed by the scientific community. Overall, many of the biochemical features that have been observed in dying trypanosomatids such as the activation of lysosomal proteases (cathepsins), ROS over-generation and the absence of caspase involvement are characteristic of a cell death close to necrosis as defined by the NCCD.\textsuperscript{14} On the other hand the pre-mentioned morphological features acquired during this form of death are characteristic of apoptosis. This questions the paradigmatic opposition between apoptosis and necrosis,\textsuperscript{35} as it proves that a cell does not require the canonical mediators of apoptosis (i.e. caspase activation) to have an apoptotic morphology.

If the apoptosis versus necrosis outcome of the death process does not always depend on the activation/presence of the complex network of canonical mediators, then what determines the outcome of a death process? A possible answer has been provided by Kroemer, who suggested that the outcome of cell death depends on the intensity of mitochondrial dysfunction.\textsuperscript{35}

If mitochondrial dysfunction is massive to heavily compromise the cell's ATP supply, the outcome is necrosis, whereas a milder form of mitochondrial deregulation results in apoptosis.\textsuperscript{35} However, further investigation is required to identify the determinants of the apoptotic/necrotic switch.

Importantly, the redundancy of canonical mediators of apoptosis to induce a typical apoptotic morphology is not only true for primitive eukaryotic cells, but also for mammalian cells.\textsuperscript{36} This was demonstrated by the discovery of a caspase-independent programmed cell death, realized in multicellular lineages by mitochondrial proapoptotic proteins.\textsuperscript{37-39} This process is reminiscent of the form of ‘apoptosis’ that takes place in unicellular eukaryotes. In addition to the existence of a caspase-independent pathway of cell death, investigators have shown that mice lacking the key elements of the core machinery of apoptosis (multiple caspase mutants, \textit{apaf-1\textsuperscript{−/−}}, or \textit{bax\textsuperscript{−/−}} or \textit{bak\textsuperscript{−/−}} double knockout mice), could survive embryonic development and develop into largely normal adulthood.\textsuperscript{36,40} This suggests that the contribution of death processes mediated by non-canonical regulators may have been underscored in mammalian cells during development and tissue homeostasis, and that the understanding of eukaryotic non-canonical cell death pathways might provide scientists answers to yet unresolved questions. The non-canonical cell death pathways in both metazoan and protozoan cells indicate that the clear distinction between necrosis and apoptosis is artificial, suggesting that the existing opinion on cell death must be modified.

Despite the complexities of death programs, a classification of the death process in unicellular organisms and how this is linked to the metazoan death programs in terms of functionality is crucial. Nevertheless, researchers should have in mind the danger of becoming fixated on death programs and of trying to match what they see to what is already known, as this might lead to suboptimal predictions of what is occurring in unicellular eukaryotes.

Overall, the occurrence of a death pathway with typical apoptotic features, but lack of canonical regulators in trypanosomatids and other unicellular eukaryotes, but also the identification of ‘non-canonical’ cell death mechanisms in metazoan cells with the same outcomes to ‘canonical’ apoptosis, is a challenge. Its clarification will provide us with both an evolutionary explanation and a framework to refer to. This is one of the specific objectives of the European Cooperation in the field of Scientific and Technical Research action BM0802 “Life and death of protozoan parasites.”

Trypanosomatids, one of the earliest branching group of eukaryotes, represent a simple and valuable model the use of which will assist in the future understanding of the complex connections between apoptotic and non-apoptotic mammalian cell death pathways. Finally, further elucidation of pathways and proteins that contribute to the regulated death of these protozoa, will shed more light to the evolutionary origins of cell death in eukaryotic cells and will provide us with tools to develop more effective drugs for combating the devastating diseases caused by these parasites.

Acknowledgments

The authors are members of COST (European Cooperation in the field of Scientific and Technical Research) action BM0802 “Life and Death of Protozoan Parasites” and appreciate support from this action.

References

1. Lockshin RA, Facey CO, Zakeri Z. Cell death in the heart. Cardiol Clin 2001; 19:1-11.
2. Smiric D, Duszynski M, Juanes-Ruiz AJ, Soudlica E, Bastien P, Fasel N, et al. Targeting essential pathways in trypanosomatids gives insights into protozoan mechanisms of cell death. Parasit Vectors 2010; 3:107.
3. Knoll AH. The early evolution of eukaryotes: a geological perspective. Science 1992; 256:622-7.
4. Stuurt K, Brun R, Croft S, Faihlamb A, Gurtler RE, McKerrow J, et al. Kinetoplastids: related protozoan pathogens, different diseases. J Clin Invest 2008; 118:1301-10.
5. Welburn SC, Maudlin I. Control of \textit{Trypanosoma brucei brucei} infections in tsetse, Glossina morsitans. Med Vet Entomol 1997; 11:286-9.
6. Welburn SC, Maudlin I, Ellis DS. Rate of trypanosome killing by lectins in midguts of different species and strains of Glossina. Med Vet Entomol 1989; 3:77-82.
7. Figarella K, Rawer M, Uczategui NL, Kubarta BK, Lauber K, Madeo F, et al. Prostaglandin D2 induces programmed cell death in \textit{Trypanosoma brucei brucei} bloodstream form. Cell Death Differ 2005; 12:395-46.
8. Luder CG, Campuz-Salinas J, Gonzalez-Rey E, van Zandbergen G. Impact of protozoan cell death on parasite-host interactions and pathogenesis. Parasit Vectors 2010; 3:116.
9. van Zandbergen G, Bollinger A, Wenzel A, Kamhawi S, Voll R, Klinger M, et al. Leishmania disease development depends on the presence of apoptotic promastigotes in the virulent inoculum. Proc Nail Acad Sci USA 2006; 103:13837-42.
10. Nguews PA, Ferrer MA, Valladares B, Alonso C, Perez JM. Programmed cell death in trypanosomatids: a way to maximize their biological fitness? Trends Parasitol 2004; 20:375-80.
11. Ameisen JC. On the origin, evolution, and nature of programmed cell death: a timeline of four billion years. Cell Death Differ 2002; 9:367-93.
12. Nedelcu AM, Driscoll WW, Durand PM, Herron MD, Rashidi A. On the paradigm of altruistic suicide in the unicellular world. Evolution 2011; 65:3-20.
13. Pollirt LC, Colegravte N, Khan SM, Sajid M, Reece SE. Investigating the evolution of apoptosis in malaria parasites: the importance of ecology. Parasit Vectors 2010; 3:105.
14. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 2009; 16:3-11.
15. Jimenez-Ruiz A, Alcace LF, Maclennan TD, Luder CG, Fasel N, Hurd H. Apoptotic markers in protozoan parasites. Parasit Vectors 2011; 4: 304.

16. Kaczanowski S, Sajid M, Reece SE. Evolution of apoptosis like programmed cell death in unicellular protozoan parasites. Parasit Vectors 2011; 4: 44.

17. Kosic G, Alvarez VE, Aguro F, Sanchez D, Dolinar M, Turk B, et al. Metacaspases of Trypanosoma cruzi: possible candidates for programmed cell death mediators. Mol Biochem Parasitol 2006; 145: 18-28.

18. Szallies A, Kubata BK, Duszenko M. A metacaspase of Trypanosoma brucei causes loss of respiration competence and clonal death in the yeast Saccharomyces cerevisiae. FEBS Lett 2002; 517: 144-50.

19. Zalila H, Gonzalez IJ, El-Fadili AK, Delgado MB, Desponts C, Schaff C, et al. Processing of metacaspase into a cytoplasmic catalytic domain mediating cell death in Leishmania major. Mol Microbiol 2011; 79: 222-39.

20. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 2001; 412: 95-9.

21. Gannavaram S, Vedyas C, Debrabant A. Conservation of the pro-apoptotic nuclease activity of endonuclease G in unicellular trypanosomatid parasites. J Cell Sci 2008; 121: 99-109.

22. Rico E, Alzaete JF, Arias AA, Moreno D, Clot J, Gago F, et al. Leishmania infantum expresses a mitochondrial nuclease homologous to EndoG that migrates to the nucleus in response to an apoptotic stimulus. Mol Biochem Parasitol 2009; 163: 28-38.

23. Bose-Daity Gupta S, Das BB, Sengupta S, Ganguly A, Roy A, Dey S, et al. The caspase-independent mechanism of programmed cell death in Leishmania induced by bafilomycin: the role of LdEndoG, LdFEN-1 and LdTadD as a DNA ‘degradesome’. Cell Death Differ 2008; 15: 1629-40.

24. Das R, Roy A, Dutta N, Majumder HK. Reactive oxygen species and imbalance of calcium homeostasis contribute to curcumin induced programmed cell death in Leishmania donovani. Apoptosis 2008; 13: 867-82.

25. Sen N, Das BB, Ganguly A, Mukherjee T, Tripathi G, Bandyopadhyay S, et al. Camptothecin induced mitochondrial dysfunction leading to programmed cell death in unicellular hemoflagellate Leishmania donovani. Cell Death Differ 2004; 11: 924-36.

26. Das M, Mukherjee SB, Shaha C. Hydrogen peroxide induces apoptosis-like death in Leishmania donovani promastigotes. J Cell Sci 2001; 114: 2461-9.

27. Irigoin F, Inada NM, Fernandes MP, Picenza L, Gadelha FR, Vercesi AE, et al. Mitochondrial calcium overload triggers complement-dependent superoxide-mediated programmed cell death in Trypanosoma cruzi. Biochem J 2009; 418: 595-604.

28. Sen N, Das BB, Ganguly A, Mukherjee T, Bandyopadhyay S, Majumder HK. Camptothecin-induced imbalance in intracellular cation homeostasis regulates programmed cell death in unicellular hemoflagellate Leishmania donovani. J Biol Chem 2004; 279: 52366-75.

29. Reape TJ, McCabe PF. Apoptotic-like regulation of programmed cell death in plants. Apoptosis 2010; 15: 249-56.

30. Mehran A, Shaha C. Apoptotic death in Leishmania donovani promastigotes in response to respiratory chain inhibition: complex II inhibition results in increased pentamidine cytotoxicity. J Biol Chem 2004; 279: 11798-813.

31. Blackstone NW, Green DR. The evolution of a mechanism of cell suicide. Biosci Rep 1999; 21: 84-8.

32. Koosin IY, Avravind L. Origin and evolution of eukaryotic apoptosis: the bacterial connection. Cell Death Differ 2002; 9: 394-404.

33. Nedelcu AM. Comparative genomics of phylogenetically diverse unicellular eukaryotes provide new insights into the genetic basis for the evolution of the programmed cell death machinery. J Mol Evol 2009; 68: 256-68.

34. Frade JM, Michaelidis TM. Origin of eukaryotic programmed cell death: a consequence of aerobic metabolism? Bioessays 1997; 19: 827-32.

35. Kroemer G. Mitochondrial implication in apoptosis. Towards an endosymbiotic hypothesis of apoptosis evolution. Cell Death Differ 1997; 4: 443-56.

36. Yuan J, Kroemer G. Alternative cell death mechanisms in development and beyond. Genes Dev 2004; 20: 2592-602.

37. Lorenzo HK, Susin SA, Penninger J, Kroemer G. Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell Death Differ 1999; 6: 516-24.

38. Mishra NC, Kumar S. Apoptosis: a mitochondrial perspective on cell death. Indian J Exp Biol 2005; 43: 25-34.

39. Susin SA, Daugas E, Ravagnan L, Samejima K, Zamzami N, Loeffler M, et al. Two distinct pathways leading to nuclear apoptosis. J Exp Med 2000; 192: 571-80.

40. Chauhan M, Chazal G, Cecconi F, Gruss P, Golstein P. Interdigital cell death can occur through a necrotic and caspase-independent pathway. Curr Biol 1999; 9: 967-70.

41. Lindsten T, Thompson CB. Cell death in the absence of Bax and Bak. Cell Death Differ 2006; 13: 1272-6.

42. Lazarova A, Aidinis V, Olive PJ, Reed JC. Apoptosis: the X-chromosome gene AIF mediates a mitochondrial pathway of cell death. Cell 1999; 96: 481-90.