Programmed cell death in *Leishmania*: biochemical evidence and role in parasite infectivity

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Demonstration of features of a programmed cell death (PCD) pathway in protozoan parasites initiated a great deal of interest and debate in the field of molecular parasitology. Several of the markers typical of mammalian apoptosis have been shown in *Leishmania* which suggested the existence of an apoptosis like death in these organisms. However, studies to elucidate the downstream events associated with phosphotidyl serine exposure, loss of mitochondrial membrane potential, cytochrome c release, and caspase-like activities in cells undergoing such cell death remain an ongoing challenge. Recent advances in genome sequencing, chemical biology should help to solve some of these challenges. *Leishmania* genetic mutants that lack putative regulators/effectors of PCD pathway should not only help to demonstrate the mechanisms of PCD but also provide tools to better understand the putative role for this pathway in population control and in the establishment of a successful infection of the host.

Keywords: apoptosis, endonuclease, *Leishmania*, metacaspase, programmed cell death, protozoa, trypanosomatid

INTRODUCTION

Programmed cell death (PCD), commonly manifested as apoptosis, plays crucial roles in a multitude of physiological processes starting from embryogenesis to maintenance of the immune system. Evolutionarily, apoptosis emerged along with multicellular organisms, primarily as a host defense mechanism against viral infections (Ameisen, 1996). However, since the first description of a PCD-like pathway in trypanosomatid parasites (Ameisen et al., 1995), increasing experimental evidence has accumulated suggesting that similar processes also appear to exist in many single-celled parasitic organisms including *Plasmodium* species (Al-Olayan et al., 2002; Ch'ng et al., 2010), *Toxoplasma gondii* (Peng et al., 2003), *Trichomonas vaginalis* (Chose et al., 2002), *Entamoeba histolytica* (Villalba et al., 2007), and *Giardia lamblia* (Ghosh et al., 2009). In trypanosomatids, features suggesting PCD have been reported in response to a wide range of stimuli such as heat shock, reactive oxygen species, antiparasitic drugs, prostaglandins, and antimicrobial peptides (Lee et al., 2002; Duszenko et al., 2006; Jimenez-Ruiz et al., 2010). Many biochemical events that accompany mammalian apoptosis such as generation of reactive oxygen species, increase in cytosolic Ca^{2+} levels, alterations in mitochondrial outer membrane potential, exposure of phosphatidylserine (PS) in the outer leaflet of the plasma membrane, release of cytochrome c, activation of caspase-like activities and nucleases that cleave genomic DNA have also been widely documented in trypanosomatid parasites (Sereno et al., 2001; Arnoult et al., 2002; Lee et al., 2002; Mukherjee et al., 2002; Zangger et al., 2002; Debrabant et al., 2003; van Zandbergen et al., 2010). Although autophagy-related processes typically used

by cells as a survival mechanism in response to stress have also been shown to lead to cell death under certain conditions (Debnath et al., 2005), their contribution to PCD in parasitic protozoan remains to be elucidated. Therefore, this review will only focus on the evidence for a PCD pathway in *Leishmania* and review the putative molecules involved in such pathway, if adequately demonstrated. We discuss the putative role of PCD in *Leishmania* infectivity and suggest future approaches to better understand the role of such cell death pathway in *Leishmania* and related trypanosomatid parasites.

BIOCHEMICAL EVIDENCE OF PROGRAMMED CELL DEATH IN *Leishmania*

MORPHOLOGICAL CHANGES

In metazoan organisms, cell morphology during execution phase of apoptosis is accompanied by characteristic changes that are distinct from other forms of cell death namely autophagy and necrosis (Klionsky et al., 2008; Kroemer et al., 2009; Galluzzi et al., 2012). Loss of cell volume with an intact plasma membrane is considered a hallmark of a cell undergoing apoptotic death unlike necrotic death where such loss of volume is usually a result of non-intact plasma membrane (Kroemer et al., 2009; Galluzzi et al., 2012). *Leishmania* parasites go through a series of distinct morphological shapes and sizes during their life cycle in the insect vector and mammalian hosts. These distinct developmental stages during the normal differentiation of the parasite have been well-characterized (Handman, 1999; Gossage et al., 2003). During its differentiation from procyclic to metacyclic promastigotes in the sand fly vector, the body of the parasite undergoes dramatic

shrinkage which is associated with autophagic processes (Besteiro et al., 2006) that do not culminate in cell death. However, the morphological changes observed during *Leishmania* PCD (e.g., cell shrinkage, nuclear condensation) are not well understood; therefore, unlike for metazoans, cell shrinkage cannot be used as a reliable marker of PCD in these organisms.

PHOSPHATIDYLSERINE EXPOSURE AT THE CELL SURFACE

Phospholipid composition in the plasma membrane of mammalian cells is not identical between the two leaflets of the membrane bilayer. The outer leaflet is predominantly composed of choline-containing phospholipids, phosphatidylcholine, and sphingomyelin, whereas the aminophospholipids, phosphatidylethanolamine, and PS populate the inner leaflet (Bevers and Williamson, 2010). This asymmetry in the lipid composition is maintained in quiescent cells by an ATP-dependent mechanism (Tang et al., 1996). However, in apoptotic cells such asymmetry is lost and as a result PS is exposed at the cell surface that can be detected by its reactivity with annexin-V (Martin et al., 1995). This PS exposure was identified as an early event in cells undergoing apoptosis regardless of the stimuli in mammalian apoptosis. Several studies in *Leishmania* reported PS exposure in stationary phase promastigotes and also in response to heat shock, serum deprivation, and a range of chemical inducers based on annexin-V binding to these parasites which is widely used maker of PCD in these organisms (de Freitas Balanco et al., 2001; Jimenez-Ruiz et al., 2010). Moreover, PS-dependent recognition and engulfment of *Leishmania* parasites by mammalian phagocytic host cells have been proposed as a mechanism for invading macrophages and in inducing an anti-inflammatory response by macrophages and dendritic cells (Wanderley et al., 2006). Recently, exposure of PS on *L. amazonensis* parasites derived from skin lesions has been shown to correlate with diffuse cutaneous leishmaniasis compared to localized lesions (França-Costa et al., 2012). Similar PS exposure–dependent mechanism is also utilized by vaccinia virus to enter host cells (Mercer and Helenius, 2008). In mammalian cells, membrane bound protein(s) are considered essential for exposure of PS as a result of collapsed asymmetry in lipid distribution and the protein likely responsible for this membrane scrambling activity was identified as phospholipid scramblase1 (Bevers and Williamson, 2010). However, *Leishmania* and other trypanosomatid parasite genomes do not have an easily identifiable sequence homolog of this protein, underlining the importance of further studies to assess the mechanism of PS exposure to the cell surface of *Leishmania*. In addition, annexin-V is also known to bind anionic phospholipids other than PS. Reagents that could specifically react with PS such as PS-specific monoclonal antibodies or molecular pattern recognition reagents such as aptamers may be of value in confirming that PS is indeed expressed on the cell surface of *Leishmania*.

CYTOCHROME C RELEASE

In mammalian cells, two major signaling cascades lead to apoptosis. The extrinsic cascade is mediated by activation of tumor necrosis family receptors, also known as death receptors. In contrast, the intrinsic cell death pathway is triggered by stimuli such as cytokine deprivation, DNA damage, and cytotoxic stress

and is orchestrated by mitochondria (Brenner and Mak, 2009). The pro-apoptotic Bcl-2 members Bax, Bak, Bid, and others initiate the mitochondrial cell death pathway by permeabilizing the mitochondrial outer membrane. This allows the release of proapoptotic factors from the mitochondria such as cytochrome c, which binds to the adaptor protein Apaf-1 resulting in the activation of caspase-9 (Brenner and Mak, 2009; Pradelli et al., 2010; Abdelwahid et al., 2011; Huttemann et al., 2011). In addition, mitochondrial alterations including disruption of electron transport, oxidative phosphorylation and ATP production, release of other proteins such as Htr2/Omi, Smac/Diablo that trigger caspase activation and changes of cellular redox potential also contribute to the intrinsic PCD pathway (Green and Reed, 1998; Pradelli et al., 2010). Release of cytochrome c has been well documented in many species of *Leishmania* in response to several apoptotic stimuli (Gannavaram et al., 2008; Jimenez-Ruiz et al., 2010). This was also observed when the proapoptotic mammalian Bax protein was exogenously expressed in *Trypanosoma brucei* (Esseiva et al., 2004). However, release of cytochrome c in isolation may be of limited utility as a definite marker of *Leishmania* PCD unless downstream events are better understood. This is because even though cytochrome c is released from the inter membrane space of mitochondria into the cytoplasmic compartment of *Leishmania*, no evidence for downstream events such as binding to Apaf- 1 and activation of caspase-9 homologs has been demonstrated. Homology searches in the *Leishmania* genome databases indicate the presence of a possible homologue of Apaf-1 protein (**Table 1**). It would be interesting to investigate if this protein indeed has Apaf-1 activity (i.e., bind to *Leishmania* cytochrome c and promote cell death). Mouse genetic studies have shown that Lys72 of cytochrome c is essential for its interaction with Apaf-1, and a mutant lacking the Lys72 can still function in electron transport chain (Hao et al., 2005). However, in a knock-in mouse with this mutation even though Apaf-1 oligomerization did not occur, caspase activation indeed took place suggesting that Apaf-1 may only speed up apoptosis but is not absolutely required for apoptosis to proceed further (Ekert et al., 2004). On the other hand, in yeast, cytochrome c release from mitochondria has been suggested to promote cell death although causal explanation is lacking (Ludovico et al., 2002). These reports suggest that similar to yeast, the release of cytochrome c in *Leishmania* needs further investigation in order to identify the signaling events associated with this molecule that are involved in PCD. This would help to define further a putative intrinsic PCD pathway in *Leishmania*.

CASPASE-LIKE AND METACASPASE ACTIVITIES

Caspases and the members of the Bcl-2 family are the most important regulators of the apoptotic process in metazoans. In protozoan parasites, however, there is very little information about the existence of homologs of the Bcl-2 proteins, even though some indirect evidence indicates that Bcl2-responsive proteins may exist in *Leishmania* (Alzate et al., 2006). On the other hand, extensive evidence for the existence of caspase-like activities associated with parasite PCD has been published (Lee et al., 2002; Paris et al., 2004; Sen et al., 2004; Singh et al., 2005). These groups have reported the activation of proteases

Table 1 | We searched the literature and conducted BLASTP homology searches, followed by reciprocal best-hit analysis, to assemble a list of
***Leishmania* homologs of the putative regulators or effectors of PCD.**

Human homolog (UniProt)	BLASTP reciprocal best-hit	High-score	Expectation value	Reference(s)
Endonuclease G (Q14249)	LmjF.10.0610	150	4.1e-22	Gannavaram et al., 2008
	LinJ.10.0660	147	3.0e-21	BoseDasgupta et al., 2008
				Rico et al., 2009
TatD deoxyribonuclease (Q6P1N9)	LmjF.11.1280	599	2.2e-59	Gannavaram and Debrabant, 2012
	LinJ.11.1270	593	9.6e-59	BoseDasgupta et al., 2008
Flap endonuclease 1 (FEN1) (P39748)	LmjF.27.0250	909	1.6e-92	BoseDasgupta et al., 2008
	LinJ.27.0260	905	4.2e-92	
Apoptosis-inducing factor 1 (AIF1) (O95831)	LmjF.32.3310	111	0.00049	none
	LinJ.32.3510	114	0.00023	
Apoptotic protease-activating factor 1 (O14727)	LmjF.10.0780	299	2.2e-25	none
	LinJ.10.0830	303	5.1e-26	

We identified homologs for five of such regulators or effectors of PCD. Some of these molecules have been characterized in terms of their biochemical activities and role in PCD but others need future studies. We did not identify homologs for any of the other mitochondrial or other organelles based molecules with roles in metazoan apoptosis (http://stke.sciencemag.org/cgi/cm/stkecm;CMP_18019; Bergmann and Steller, 2010).

able to degrade classical substrates of mammalian caspases in *Leishmania* undergoing PCD. Even though caspase-like activities have been repeatedly reported these protease activities do not appear to be due to bona fide caspases, due to the fact that "caspase" genes have not been found in any of the available complete *Leishmania* genomes and none of the genes encoding the caspase-like enzymatic activities reported in *Leishmania* have been cloned.

Orthologs of caspases, i.e., metacaspases (MCs) and paracaspases, have been identified first from slime mold and later from several plants (Uren et al., 2000). MCs are cysteine proteases with structural similarity to caspases containing a catalytic cysteine histidine dyad. However, biochemical analyses revealed that MCs have distinct substrate specificity (Lys/Arg) from caspases (Asp). A role for MCs in PCD has been reported in plants, yeasts, and protozoan parasites including *Leishmania* (Silva et al., 2005; Lee et al., 2007; Meslin et al., 2007; Coll et al., 2010). Most *Leishmania* species contain a single MC gene except in *L. infantum* and *L. donovani* subtypes where two MCgenes have been found (Lee et al., 2007). Structurally, *Leishmania* MCs contain a central active domain containing the conserved catalytic cysteine and histidine dyad and a proline-rich C-terminal domain (Lee et al., 2007). In *L. donovani*, LdMC1 and LdMC2 cleave arginine/lysine containing substrates without any requirement for proteolytic activation, neither in normal conditions nor upon oxidative stress-induced PCD. LdMCs were reported to be stored in acidocalcisomes and released from these vesicles when cells were treated with hydrogen peroxide (Lee et al., 2007). Further, *Leishmania* overexpressing LdMC1 were more sensitive to hydrogen peroxide treatment suggesting a role in PCD (Lee et al., 2007). In contrast to LdMCs, the *L. major* MC (LmjMC) was reported to be activated by autoprocessing and its recombinant putative catalytic domain was ~300 times more active than the full-length recombinant enzyme for

cleaving a fluorogenic substrate containing GGR residues *in vitro* (Gonzalez et al., 2007). LmjMC overexpression was found to enhance *L. major* sensitivity to oxidative stress as measured by the increase of phosphotidyl serine exposure at the parasites surface and the rapid loss of mitochondrial membrane potential, also suggesting a role in *Leishmania* PCD (Gonzalez et al., 2007; Zalila et al., 2011). However, the physiological substrates of *Leishmania* MCs remain to be identified. Interestingly, similar to *Leishmania, Plasmodium* MC has also been shown to exhibit a calcium-dependent, arginine-specific protease activity. Further expression of *P. falciparum* metacaspase in the yeast MC null mutant increased its susceptibility to undergo cell death under oxidative stress (Meslin et al., 2011). A promising approach that could yield valuable information regarding proteins that are substrates for MCs is the activity based probes that have been successfully employed in characterizing several cysteine proteases (Bogyo et al., 2000), kinases (Cohen et al., 2005), and phosphatases (Kumar et al., 2004). Identification of cellular substrates is the first step in further elucidating the role of MC in *Leishmania* PCD. A non-apoptotic role has also been ascribed to LmjMC. LmjMC was found to play a role during organelle segregation and cell-cycle progression under normal physiological conditions (Ambit et al., 2008). In addition, LmjMC null mutants were only viable when LmjMC was re-expressed from an episome at physiological levels, thus reinforcing its importance in parasite survival (Ambit et al., 2008). These observations suggest that *Leishmania* MCs could have separate roles depending on the environmental conditions. Additional studies will be needed to better understand the various roles of MCs in *Leishmania*.

NUCLEASE ACTIVITIES

Among the putative effector molecules of *Leishmania* PCD, nucleases have been best characterized. Since no bona fide caspase

has been detected in trypanosomatid parasite, caspase-dependent DNase is unlikely to be encoded by their genomes as evidenced by the absence of any homolog of caspase-activated DNase (CAD) in their genomes. In mammalian apoptosis, two endonucleases are known to be involved in DNA fragmentations that do not require activation by caspases. These are apoptosis inducing factor (AIF) and endonuclease G (EndoG). EndoG has been shown to be a mitochondrial enzyme that is released in response to inducers of apoptosis and is involved in DNA degradation of dying cells (Li et al., 2001). In *Leishmania*, EndoG-mediated DNA degradation has been independently demonstrated in three different laboratories using hydrogen peroxide or pharmacological agents (BoseDasgupta et al., 2008; Gannavaram et al., 2008; Rico et al., 2009). The overexpression of EndoG in *Leishmania* resulted in spontaneous DNA fragmentation in amastigotes and not in the promastigotes, suggesting that additional factors necessary for efficient DNA degradation are expressed in a stage-specific manner (Gannavaram et al., 2008). In *L. major*, oxidative stress associated with the differentiation process induced high levels of PCD when EndoG is overexpressed in *L. major* parasites (Gannavaram et al., 2008). Also, induction of oxidative burst in macrophages by LPS and IFN-γ treatment triggered PCD in intracellular amastigotes compared to non-stimulated host cells indicating that oxidative burst can induce PCD even in wild type parasites. These results suggested a role for EndoG in *Leishmania* PCD.

Functional genomic studies in *C. elegans* showed that several nucleases are involved in DNA degradation observed during apoptosis (Parrish and Xue, 2003). They include nuc-1, cps-6, AIF, cell death-related nucleases 1–6, and cyclophilin-13 (Parrish et al., 2001; Wang et al., 2002; Parrish and Xue, 2003). In *Leishmania*, indirect evidence is presented for a role of additional nucleases such as TatD-related nuclease and flap endonuclease in DNA degradation during PCD (BoseDasgupta et al., 2008). Recently, we described the involvement of TatD nuclease during PCD in the protozoan parasite *Trypanosoma brucei*. *T. brucei* TatD nuclease showed intrinsic DNase activity was localized in the cytoplasm and translocated to the nucleus where it could interact with EndoG when cells were treated with inducers that cause PCD. Over-expression of TatD nuclease resulted in elevated PCD and conversely, loss of TatD expression by RNAi conferred significant resistance to the induction of PCD in *T. brucei* (Gannavaram and Debrabant, 2012). These results show that *T. brucei* overexpressing TatD spontaneously undergo PCD under conditions that the parasites are likely to encounter in a human host, supporting a role of TatD in PCD in trypanosomatid parasites.

An AIF homolog has been shown to be translocated from the mitochondria to the nucleus after the onset of PCD in *Dictyostelium discoideum* (Arnoult et al., 2001). Recent searches in the *Leishmania* genomes suggested the presence of a weak homolog of AIF (**Table 1**). It would be of interest to investigate if this molecule has nuclease activity associated with PCD. Additionally, a recent report demonstrated in *C. elegans* the role of DICER in mediating DNA strand breaks during cell death associated with the normal development of this organism (Nakagawa et al., 2010). DICER usually degrades double stranded RNA into small RNAs that are involved in gene silencing. The unusual

change in substrate specificity of DICER from a dsRNA to a DNA is accompanied by proteolytic cleavage of its carboxy terminal region (Nakagawa et al., 2010). In comparison to other eukaryotic cells, most *Leishmania* species are known to have lost the machinery required for dsRNA-mediated RNA interference except *L. braziliensis* and other species within the *Leishmania* subgenus *Viannia* (Lye et al., 2010). It would be of interest to investigate if *L. braziliensis* DICER has DNase activity that is pertinent to PCD. In addition, since DICER activity has also been demonstrated in *T. brucei* (Shi et al., 2006), the putative role of this molecule in *T. brucei* PCD could be an interesting avenue to pursue.

ROLE OF *Leishmania* PCD IN PARASITE INFECTIVITY AND SURVIVAL

POPULATION CONTROL

Several arguments have been made recently about the existence of a PCD pathway and the physiological functions that such pathway might serve in the life cycle of unicellular parasites. For instance, PCD is considered useful in regulating the parasite cell density in the host as a mean of avoiding hyperparasitism. In *Plasmodium,* a PCD-like mechanism was suggested as a modality for limiting parasite density in the mosquito and in the mammalian hosts (Al-Olayan et al., 2002; Deponte and Becker, 2004). *T. brucei* parasites have been hypothesized to utilize PCD to regulate their cell densities in the insect vector and in mammalian host in response to the changing host antibody repertoire because of the variations in antigenic composition on its surface (Welburn and Maudlin, 1997; Vassella et al., 1997). Further, it has been shown that addition of prostaglandin D2 to cultures inhibited growth of bloodstream form parasites and induced an apoptosis-like cell death (Figarella et al., 2005). These observations led to the hypothesis that the high serum prostaglandin levels in the mammalian host may play a role in regulating parasite densities by inducing cell death (Figarella et al., 2005; Duszenko et al., 2006). However, definitive evidence that links the role of PCD in controlling parasite density in either mammalian or invertebrate hosts for *Plasmodium* or *T. brucei* is lacking. Similar evidence demonstrating a role for PCD in the regulation of parasite population is also lacking in *Leishmania* even though apoptotic features have been widely demonstrated in these parasites (Luder et al., 2010). To that end, the development of genetic mutants lacking the putative regulators/effectors of *Leishmania* PCD described so far (e.g., MC or EndoG null mutants) would be extremely useful to evaluate the role of parasite PCD in maintaining host–parasite equilibrium.

MODULATION OF HOST IMMUNITY

PCD has also been considered to influence the outcome of an infection during the early phase of interactions between parasites and their mammalian host. Recently it has been proposed that for establishing a successful *L. major* infection in mice, the presence of apoptotic parasites in the inoculum was a key determinant (van Zandbergen et al., 2006, 2010). This was based on the observation that presence of annexin-V binding parasites within the inoculum leads to increased uptake by neutrophils that are attracted to the site of infection before the macrophages

home in van Zandbergen et al. (2004, 2006). This was accompanied by the release of anti-inflammatory cytokines such as TGF-β, IL-10 and lipids such as lipoxinA4 and down-regulation of pro-inflammatory cytokines such as TNF-α and lipids like leukotriene-B4, which could favor parasite survival. In addition, in the *L. major* infection, when the annexin-V binding parasites were depleted from the inoculum, the presumably non-apoptotic parasites had limited virulence as indicated by the reduced size of the lesions. Further, the release of the anti-inflammatory cytokine TGF-β by the neutrophils correlated with the dose of the annexin-V binding parasites in the inoculum. Also there was an inverse correlation between the annexin-V binding parasites and the secretion of pro-inflammatory cytokine TNF-α. (van Zandbergen et al., 2006). These observations led to the hypothesis that the "eat-me" signal represented by the annexin-V binding on the parasites surface promotes immunologically "silent" uptake of the *Leishmania* parasites by macrophages and dendritic cells, as was observed in other immunosuppressive effects of apoptotic cells in mammalian homeostatic cell death (Huynh et al., 2002; van Zandbergen et al., 2006; Obeid et al., 2007). For this silent uptake to happen, the parasites in the inoculum must be able to recruit phagocytic cells to the inoculation site since the phagocytes require chemotactic guidance. There is some evidence to indicate that neutrophils are recruited by a *Leishmania* chemotactic factor and this activity is likely present in the lipid fraction of the parasites (van Zandbergen et al., 2007). Additional chemotactic signals such as lysophosphatidylcholine, sphingosine 1-phosphate and CX3CL1/fractalkine have been described for their potential to recruit professional phagocytes such as macrophages (Li, 2012). However, roles of such additional "find me" signals in the context of a *Leishmania* infection remain to be investigated. In a *Leishmania* infection, neutrophils migrate to the site within 40 min and localize around bite sites (Peters et al., 2008). The neutrophils recognize the phosphotidyl serine exposed on a subpopulation in the metacyclics and phagocytose the cells in the inoculum in a non-immunogenic mechanism.

Recently, calreticulin, mostly know as an endoplasmic reticulum (ER) chaperone protein, was shown to also function as "eat me" signals (Obeid et al., 2007). Calreticulin was shown to be upregulated on the surface of apoptotic cancer cells that favored their uptake by phagocytic cells in a non-immunogenic "silent" mechanism (Obeid et al., 2007; Martins et al., 2010). The role of calreticulin as an ER chaperone molecule has been previously characterized in *Leishmania* (Debrabant et al., 2002). Whether this protein is also exposed at the surface of *Leishmania* to facilitate their silent entry into the host phagocytic cells needs to be investigated. Additionally, mannose binding lectins or lung surfactant proteins A and D have also been shown to act as eat-me signals (Ogden et al., 2001; Vandivier et al., 2002). In contrast, negative regulators of phagocytosis such as lactoferrin have also been described (Bournazou et al., 2009). All this diversity of signals indicates that phagocytosis is a finely regulated process with broad physiological effects and in principle parasites might utilize many signals to gain silent entry into a host cell. Therefore, it would be pertinent to explore such signaling pathways that promote non-immunogenic uptake of *Leishmania*.

FUTURE PERSPECTIVES

Analysis of genetic mutants facilitated robust demonstration of the existence of genetically PCD pathways in multicellular organisms and retention of those pathways in multicellular organisms is clearly understood from an evolutionary stand point. However, the selection of a PCD pathway in unicellular organisms such as *Leishmania* is less clear and remains to be explained from an evolutionary point of view. Studies in yeast suggested arguments in support of a PCD pathway in single cell organisms. For instance, in co-culture experiments, yeast persistently infected with dsRNA viruses were shown to induce PCD in uninfected yeast cells (Ivanovska and Hardwick, 2005; Schmitt and Reiter, 2008). PCD did not occur in a yeast mutant lacking MC, demonstrating the existence of this conserved cell death pathway in a single cell organism. Recently, *Leishmania* parasites infected with a dsRNA virus have been shown to cause metastatic spreading of the otherwise localized cutaneous lesions (Ives et al., 2011). This metastasis involved modulation of host immunity by viral RNA that resulted in heightened host pro-inflammatory responses (Ronet et al., 2011). Studies in virally infected *Leishmania* parasites analogous to those in yeast might help clarify the existence of a PCD pathway in *Leishmania* parasites and support its selection in single cell organisms.

In comparison to *C. elegans* and yeast, studies elucidating molecular mechanisms of PCD in trypanosomatid parasites are limited primarily because of the apparent absence of homologues to key regulatory or effector molecules of apoptosis in the trypanosomatid genomes that have been described in mammalian or nematode apoptosis such as Bcl-2 family members and caspases (Smirlis et al., 2010). Searches in annotated *Leishmania* genomes reveal that homology based searches offer limited clues in attempts to deciphering the mechanisms mediating PCD machinery in these parasites (**Table 1**). The absence of homologues of key regulatory or effector molecules of mammalian apoptosis in trypanosomatid parasites suggests that their PCD pathways are probably less evolved than apoptotic pathways of mammalian cells. Although still poorly understood, the existence of conserved PCD pathways in trypanosomatid parasites can provide targets for developing novel chemotherapies. Recent pharmacological studies elicited interest in several molecules with activities that trigger apoptotic death in cancerous cells as potential antiparasitic agents (Fuertes et al., 2008).

Studies with genetic mutants that lack regulators/effectors of PCD (e.g., MC or EndoG null mutants) would clarify the role of each of these proteins in *Leishmania* PCD. However, this approach is contingent upon the fact that analysis of such mutants can yield evidence for a role in PCD under appropriate settings, as the same molecule may have multiple functions in the cell including functions unrelated to PCD. For instance, EndoG null mutant mice have been shown to be associated with impaired mitochondrial respiration and increased production of reactive oxygen species, indicating that pro-apoptotic activities of this nuclease are likely to be specialized functions for this protein (David et al., 2006; McDermott-Roe et al., 2011). Similarly, studies using *Leishmania* genetic mutants lacking regulators/effectors of PCD may help better understand the role of this pathway in the survival of

the parasite population in its invertebrate host. For example, to determine if PCD of non-metacyclic *Leishmania* parasites occurs in an infected sand fly and contributes to the survival of infectious metacyclic forms which is the only stage that is transmitted and can establish a successful infection in the mammalian host and therefore ensure the propagation of the species.

In summary, our knowledge of *Leishmania* PCD is still very fragmented. In the last ten years, several putative effector molecules of such pathway have been identified and characterized to various degrees. Similar effector molecules have also been shown to be involved in PCD pathways in other protozoan parasites suggesting that a common pathway might be conserved among this group of organisms. So far molecules regulating this pathway are unknown. In *Leishmania* the mitochondria appears to play a central role in this pathway (e.g., release of cytochrome c and proapoptotic nucleases) and therefore displays similarities to the intrinsic apoptosis pathway of mammalian cells. Although experimental observations are limited, there is increasing evidence to support the idea that protozoan parasites use PCD for controlling their population in the infected host. Possibly, *Leishmania* could use PCD either in the insect vector to favor the survival of infectious metacyclic forms, or in the mammalian host to avoid hyperparasitism that would prematurely kill the host. In addition, recent evidences suggest that *Leishmania* also exploits features of PCD to facilitate its silent entry in the mammalian host and establish a successful infection. However, there is much work ahead to decipher the multiple roles played by PCD in the biology of *Leishmania*.

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