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MULTI-AUTHOR REVIEW

Apoptosis and apoptotic mimicry: the *Leishmania* connection

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Abstract Different death-styles have been described in unicellular organisms. In most cases they evolve with phenotypic features similar to apoptotic death of animal cells, such as phosphatidylserine (PS) exposure, oligonucleosomal DNA fragmentation, and loss of mitochondrial transmembrane potential, hinting that similar mechanisms operate in both situations. However, the biochemical pathways underlying death in unicellular organisms are still unclear. Host recognition of PS exposed on the surface of unicellular parasites is an important feature of the process of infection and progression of the disease. Here, we discuss data showing that entirely different mechanisms of PS exposure co-exist during the life-cycle of *Leishmania amazonensis*: in the case of promastigotes, a sub-population dies by apoptosis; in the case of amastigotes, the entire population exposes PS, not necessarily followed by apoptotic death. This phenomenon has been called apoptotic mimicry. The elusive caspase-like activities described in protozoa are also discussed.

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Introduction

It is only rather recently that studies of the various forms of cell death have reached the forefront of biomedical research. The knowledge of the biochemical basis and genetic programming underlying the control and execution of the classical and alternative death styles became an issue in cell biology, as fundamental as growth, division, and differentiation (see references in this issue). In this context, programmed death in unicellular organisms has been a matter of intense and still ongoing debate. Two excellent and comprehensive reviews were recently published [1, 2]. The first of them points to the fact that, in spite of still some uncertainty in the classification of eukaryotes as well as in defining their inter-relationships [3, 4], apoptotic phenotypes have been described in unicellular organisms assigned to all major groups of eukaryotes [1]. The second one [2] deals with the role of programmed cell death (PCD) in pathogenic trypanosomatids, agents of important tropical diseases such as Leishmaniasis and African and South American trypanosomiasis, transmitted by insect vectors and thus having to cope with environmental transitions characteristic of a digenetic organism. In a great number of examples discussed in the above-mentioned reviews, apoptotic death in unicellular organisms evolves displaying features associated with PCD in animals, such as phosphatidylserine (PS) exposure, oligonucleosomal DNA fragmentation, loss of mitochondrial transmembrane potential, and caspase-like activities, suggesting that similar mechanisms might be operational in both situations. In this

review, we will mainly focus on the similarities and differences between apoptotic death and apoptotic mimicry using the infection with *Leishmania* spp. as the core model.

Apoptotic mimicry and evasion of the host immune system

One of the most remarkable features which have evolved as part of the program of cell death by apoptosis is the ability of apoptotic cells or corpses to be efficiently internalized by phagocytes, and to actively prevent inflammation [5, 6] and immune responses [7] in the organism-bearing cells undergoing apoptotic death. Viruses and parasites represent selective forces shaping—and being shaped by—the evolution of susceptible organisms [8–10]. It is thus not a surprise that parasites and viruses take advantage of the removal system used by apoptotic cells, as a strategy for evasion from the host inflammatory and immune system. First proposed to occur with the hepatitis B virus [11] and shown to occur with amastigotes of *Leishmania amazonensis* and named apoptotic mimicry [12], such a mechanism has now also been described in infections by *Toxoplasma gondii* [13] and *Trypanosoma cruzi* [14], as well as by different viruses such as vaccinia [15], cytomegalovirus, and Lassa fever virus [16]. Apoptotic mimicry, in the situations described up to the present, is the result of the surface exposure of PS—the main apoptotic cell-associated molecular pattern (ACAMP) [17, 18]—by the pathogenic organism, without death as the necessary outcome [19]. The existence of such a parasite evasion mechanism justifies recent efforts for developing therapeutic strategies based on the use of anti-PS antibodies [16]. In the case of *T. gondii*, infective tachyzoites expose PS. Within the PS-exposing forms, a highly positive subpopulation can be detected, interpreted as being recently divided forms. The mechanism by which PS-exposing tachyzoites evade the microbicidal competence of activated macrophages is by TGFß1-dependent NO inhibition [13]. A very similar phenomenon has been described in infection with *T. cruzi* where, of all the differentiation forms of this parasite, only infective trypomastigotes expose PS. Surface PS triggers the TGFß signaling pathway evidenced by Smad2 translocation to the nucleus, leading to the disappearance of iNOS from macrophages [12]. It remains to be explained why amastigotes, also an infective form, do not expose PS. The authors speculate that they might be endowed with a different mechanism to evade macrophage-killing activity. Apoptotic mimicry has also been described in viral infections, showing that this mechanism is not restricted to protozoan parasites. Vaccinia virus induces bleb formation in host cells and is internalized via macropinocytosis. Bleb formation, endocytosis, and infection are fully dependent on the presence of exposed PS in the viral membrane [15]. Pichinde virus, an arena virus that has been adapted to produce lethal infections in guinea-pig resembling human Lassa fever, induces PS exposure in infected cells. This is reminiscent of what happens with cells infected with several different viruses, but in the present context, Pichinde virions carry PS on their envelopes. Bavituximab, a humanized anti-PS antibody, is capable of curing guinea-pigs lethally infected with Pichinde virus [16]. In addition, bavituximab has been described as also recognizing infectious VSV virions [16]. As proposed, viruses that induce PS exposure in infected cells can incorporate this phospholipid into their envelope during budding and egress. A similar mechanism can occur in viruses that acquire their envelopes from intracellular organelles. HIV-1 is such an example [20].

Apoptotic death and apoptotic mimicry

In the case of experimental disease caused by *L. amazonensis*, both apoptotic death and apoptotic mimicry co-exist and are differently generated in the two forms of the parasite: in the case of promastigotes, a sub-population exposes PS and dies by apoptosis [21]; in the case of amastigotes, the entire population carries PS on its surface and this exposure is not necessarily followed by apoptotic death [12]. In this latter situation, exposed PS is either a non-apoptotic signaling residue as shown to occur in different cell types [22–26] or amastigotes are rescued from apoptotic death by a still unknown mechanism [27]. However, the possibility of amastigotes carrying patches of host phagolysosomal membranes exposing PS, when released from their intramacrophagic niche, has not been definitively discarded. This possibility is, however, very improbable for several reasons, including the demonstration of apoptotic mimicry in other intracellular protozoan parasites, independently of the way they interact with their respective host cell, as mentioned above. Indeed, one of the morphological characteristics of amastigotes of *L. amazonensis* is a strong polarized adherence to the membrane of the vacuole on which they dwell [28, 29]. Nevertheless, it is important to stress that the concept of apoptotic mimicry does not change with any one of the alternative mechanisms of PS exposure described above.

Several lines of evidence strongly point to the active PS exposure and rescue from apoptotic death as the most probable explanation: first, the fact that amastigotes do indeed bear a potentially functional apoptotic-death machinery, as evidenced by the demonstration of apoptotic features in *Leishmania donovani* inside macrophages treated with pentavalent antimony [30], and the demonstration that amastigotes undergoes DNA cleavage when left outside macrophages at 34°C [31]; second, the differential PS exposure in amastigotes derived from sensitive

and semi-resistant strains of mice. Indeed, a very interesting feature of apoptotic mimicry in *L. amazonensis* is the fact that amastigotes derived from BALB/c mice expose significantly more PS than C57Bl/6-derived forms [32], strongly suggesting that the exposure mechanism is under control of the host adaptive immune response. This hypothesis is reinforced by the fact that amastigotes derived from immunodeficient (nude) BALB/c mice do not expose PS after 5 weeks of infection (Deolindo, Wanderley and Barcinski, unpublished observation). Most importantly, the final outcome of PS exposure, be it by a sub-population of promastigotes undergoing apoptotic death or by amastigotes mimicking apoptotic cells, is inhibition of macrophage leishmanicidal capacity.

While the knowledge of the receptors and of the coupled transduction pathways involved in the generation of anti-inflammatory mechanisms and engulfment of apoptotic animal cells engaged by PS and opsonized PS is quickly growing, the same is not true with PS on a parasite's surface. In this last situation, what has been clearly shown is that TGFβ [13, 14, 32] and IL-10 [12, 21, 32] are involved in down-regulating the host immune response and that PS-induced macropynocitosis is involved in parasite internalization [32]. It is also known that pathogens are recognized by the host innate immune system via surface ligands, collectively known as pathogen-associated molecular patterns (PAMPs) [33]. PAMPs are recognized by host cells via non-clonally expressed pattern recognition receptors (PRR) [34, 35]. For more information about the consequence of PRR signaling to cell death and the control of infection, we refer to a different review in this issue [36]. Interestingly, PS exposure by different pathogens could be conceptually understood as the appearance of a non-constitutively expressed PAMP on their surface, endowed with the capacity to change a pro-inflammatory into an anti-inflammatory host response.

Modulation and effector mechanisms of apoptotic death: the still unsolved problem of the caspase-like activities

The establishment of a productive leishmanial infection depends on the interaction of both differentiation forms of the parasite with its mammalian host [32] with the outcome depending on several factors, including the *Leishmania* species and the immunocompetence of the host. Promastigotes are the form transmitted by sandfly vectors. During migration from the gut to the vector mouth parts of the insect, promastigotes suffer complex developmental modifications which transform them from dividing procyclic forms into non-dividing infective metacyclic forms [37]. This differentiation process can be partially reproduced in axenic in vitro cultures. Following inoculation in the host skin, promastigotes interact with different cell types, including polymorphonuclear neutrophils (PMN), different sub-sets of dendritic cells, and epidermal Langerhans cells [38], and infect macrophages where they transform into the amastigote forms [39]. Interestingly, it has been described that infected PMN exposes PS and can act as "Trojan horses", transferring parasites to already inactivated macrophages [40].

Amastigote forms reside inside parasitophorous vacuoles (PV) present in macrophages, the main host cell type for parasite multiplication and the effector cell for parasite killing. PVs acquire different forms, depending on the species of the infecting parasite: parasites comprising the *Leishmania mexicana* complex, which include *L. amazonensis*, induce a marked PV expansion resulting in very large intramacrophagic vacuoles harboring a large number of parasites [41], while other *Leishmania* spp. (e.g., *L. major*) live in tight individual PVs. There is experimental evidence showing that vacuole expansion is an important variable defining growth of amastigotes of *L. amazonensis* [42]. In short, promastigotes during their very brief lifetime in the mammalian host interact with cells and molecules of the innate immune response, while amastigotes, responsible for the natural history of the disease, modulate the establishment of the adaptive immune response, which in turn defines the course of the leishmanial disease. Within this life-cycle pattern it becomes obvious that the sites for apoptosis to occur are the guts of the insect vector and host macrophages for, respectively, promastigote and amastigote forms.

In promastigotes, apoptotic death induced by host-protective agents, such as H_2O_2 as well as in-use and potentially useful anti-leishmanial drugs, has been described [2]. A different perspective is to consider apoptotic death of a sub-population of promastigotes as part of the normal differentiation process by which non-infective procyclic promastigotes turn into infective metacyclic forms (metacyclogenesis) [43, 44]. Several advantages of a step of PCD during metacyclogenesis are foreseen: avoidance of uncontrolled parasite growth and consequent death of the vector, as described to occur with *Trypanosoma brucei* in tsetse flies; selection of fittest forms for mammalian host infection [45]; and generation of PS exposing forms, capable of inactivating host macrophages and enhancing leishmanial infection when present in the virulent inoculum. This last situation has been described in infections with *L. major* and *L. amazonensis* [21, 46].

The evidence for PCD to occur as part of the metacyclogenic process is, in vivo, the demonstration in the sandfly gut of ultrastructural markers of apoptosis in promastigotes and of TUNEL labeling in metacyclic forms accumulating in the bulbous cardia region of the foregut, and, in vitro, the fact that caspase-like activities have been

detected exclusively during a very narrow time-window at the exponential- to stationary-phase transition of the promastigote's growth curve. This type of data has been obtained by two different strategies with different read-outs. The first one took advantage of the fact that, during in vitro growth, promastigotes initiate PS exposure when reaching the stationary phase of growth and begin to acquire a morphology characteristic of metacyclic forms. The addition of a pan-caspase inhibitor, at a very specific point of the growth curve, diminishes the number of PS positive parasites. In the second approach, again the addition of caspase inhibitors only at a specific time during cell growth was able to increase the number of recovered viable promastigotes in a plating-efficiency type of experiment [21]. Caspase-like activities have been described as involved in the death process of several different pathogenic unicellular organisms. Such descriptions have raised controversies and their interpretation is by no means a solved issue. This is due, in part, to the fact that caspases, as well as genes for such molecules, were not found in pathogenic protozoa. Caspase activation has been described as being at the core of apoptotic death in mammalian cells (see Schrader et al., this issue). In 2000, Uren et al. [47] using iterative database searches described a family of caspase-like proteins (characterized as metacaspases), which are present in plants, fungi, and protozoa, and bear the predicted secondary structure and the catalytic dyad histidine/cysteine characteristic of caspases. This description generated great enthusiasm and, added to the fact that metacaspase genes have been found in the genomes of pathogenic unicellular organisms (five genes in *T. brucei*, two genes in *T. cruzi* and in *L. donovani*, a single gene in *L. major*, and two genes in *P. falciparum*), metacaspases became the main candidates for being the functional homologues of animal caspases. The demonstration that metacaspases have Arg/Lys-specific activity as opposed to activity on canonical caspase substrates [48–50] frustrated such expectations. However, the amount of data in several different model systems of unicellular parasites, showing either caspase activity or inhibition of this activity by compounds designed as caspase substrates or inhibitors, cannot be discarded, in spite of the limits of the specificity of such reagents, and demands a re-interpretation. Indeed, promastigotes of *L. major* when treated with staurosporine, a protein kinase inhibitor, undergo death with features resembling mammalian cell apoptosis. The addition of a broad mammalian caspase inhibitor (BAF) and of E-64, a cysteine-proteinase inhibitor, were independently able to significantly reduce DNA degradation of the staurosporin-treated parasites, without, strangely enough, inhibiting cell death. Inhibitors targeting other proteinases, including a proteaseome inhibitor, had no preventing effect on DNA degradation [51]. Also, in promastigotes of *L. donovani* undergoing H_2O_2-induced apoptotic-like death, the number of TdT-positive cells and the amount of DNA degradation, as well as the total number of cells with altered morphology, were significantly reduced by pre-treating the cells with Z-DEVD-FMK, a mammalian caspase-3 inhibitor. In addition, a synthetic (Ac-DEVD-AFC) and a natural (PARP) caspase-3 substrate were cleaved by promastigotes treated with H_2O_2. PARP cleavage was inhibited by Z-DEVD-FMK [52]. Extending the previously referred findings, Lee et al. [53], using the cell permeable caspase-specific substrate PhiPhiLux (PPL), showed that promastigotes of *L. donovani* entering the stationary-phase of in vitro culture, or when treated with amphotericin B, suffer PCD and display, among other features of apoptosis, significant PPL cleavage. Similar results with drug treatment were obtained with axenic amastigotes. Reinforcing the caspase-like nature of PPL-cleavage, this activity was inhibited by three different caspase inhibitors (Z-VAD-FMK, Z-DEVD-FMK, and Boc-D-FMK) and not by cathepsin and calpain inhibitors [52]. It has also been shown that a DNA topoisomerase inhibitor [(3,3′-di-indolylmethane (DIM)] was able to induce PS exposure and depolarization of mitochondrial membrane potential in promastigotes of *L. donovani*, and both effects were inhibited by the addition of VAD-fmk, a pan-caspase inhibitor. Furthermore, fluorometric assays for caspase-9 and caspase-3 revealed increased activity induced by DIM and inhibition by specific substrates [54]. On the other hand, there is evidence of the involvement of metacaspases in processes not related to apoptotic death, such as control of cell proliferation and mitochondria biogenesis as demonstrated by the heterologous expression of the *T. brucei* metacaspase in *Saccharomyces cerevisiae* [55] and of cell proliferation in *L. major* [56].

Alternative strategies to define the molecular origin and the in vivo role of the described caspase-like activities in protozoa are definitely and urgently needed. The same stands for metacaspase functions. For this last one, an interesting proposal is to define their degradome specificity by employing technologies powerful enough to directly characterize their in vivo substrates on a proteome-wide scale [57]. A similar situation occurs in plants where proteases cleaving caspase substrates are required for PCD in different experimental systems, and substantial progress is being made for their identification [58, 59].

Modulation and effector mechanisms of apoptotic mimicry

With amastigotes, the situation is very different from the one described for promastigotes. They are targets of the host adaptive immune response via macrophage activation by different cytokines derived from Th1 or Th2 $CD4^+$ T

cell immune response [60–62]. Classical macrophage activation, induced by Th1-derived cytokines, depends on L-arginine cleavage by inducible NO synthase (iNOS) and consequent NO generation and leishmanicidal activity. On the contrary, in macrophages activated by Th2-derived cytokines, L-arginine is cleaved by arginase I with consequent L-ornithine generation, polyamine synthesis and leishmanial survival and proliferation [62]. Murine infection with *L. major* is the prototype model of a polarized Th1 versus Th2 immune response. BALB/c mice, a Th2 responder strain, are susceptible to the infection, while C57Bl/6 mice, as Th1 responders, are resistant to the same infection [63]. Intracellular amastigotes can die with apoptotic phenotypes in NO producing macrophages when a strong Th1 response is activated [61]. Interestingly, infection with *L. amazonensis* is not as polarized as the one induced by *L. major,* and is characterized by persistence of IFN-γ and IL-4-producing T cells during the entire course of infection [64]. *L. amazonensis* amastigotes are exquisitely resistant to macrophage activation by IFN-γ, which in certain conditions can even promote amastigote proliferation [65]. In addition, residing in enlarged PVs protects *L. amazonensis* amastigotes from oxidative stress [42]. It is tempting to hypothesize that dwelling in large PVs in macrophages activated by a mixed Th1/Th2 response diverts the response of amastigotes of *L. amazonensis* from apoptotic death to apoptotic mimicry. Indeed, preliminary evidence points to the fact that the amount of exposed PS on amastigote forms is modulated by the intensity and quality of activation of the infected macrophage by cytokines derived from $CD4^+$ T cells (Wanderley, Deolindo, Barcinski and Soong, unpublished observations). In turn, exposed PS inhibits leishmanicidal macrophage capacity at each cycle of re-infection by amastigotes. Whether this mechanism works by modulating classical or by alternative macrophage activation is still to be shown.

In conclusion, the fact that unicellular organisms are able to undergo programmed death is already a settled matter [1, 2, 66]. The remaining challenge is to define the molecular basis of the signals and receptors triggering PCD in these organisms and the biochemical basis of its execution and genetic control, as well as to gain a better insight into the selective advantages of such a process.

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