Programmed cell death in African trypanosomes

S. C. WELBURN[1]*, E. MACLEOD[1], K. FIGARELLA[2] *and* M. DUZENSKO[2]

[1] *Centre for Infectious Diseases, College of Medicine and Veterinary Medicine, University of Edinburgh, EH25 9RG*
[2] *Department of Biochemistry, University of Tubingen, Hoppe-Seyler-Str. 4, 72076 Tubingen, Germany*

SUMMARY

Until recently it had generally been assumed that apoptosis and other forms of programmed cell death evolved during evolution of the metazoans to regulate growth and development in these multicellular organisms. However, recent research is adding strength to the original phenotypic observations described almost a decade ago which indicated that some parasitic protozoa may have evolved a cell death pathway analogous to the process described as apoptosis in metazoa. Here we explore the implications of a programmed cell death pathway in the African tsetse-transmitted trypanosomes.

Key words: Apoptosis, programmed cell death, *Trypanosoma brucei*, trypanosomatids.

INTRODUCTION

One of the shibboleths of modern cell biology has been the idea that programmed cell death (PCD) evolved to meet the particular needs of multicellular life (Vaux, Haecker and Strasser, 1994; Evan, 1994). It is generally assumed that a regularized system of cell death, among other essential development programmes, appeared in phylogenesis after the onset of multicellularity. This hypothesis was not based on experimental evidence – showing that all unicellular organisms lack PCD – but was a reflection of the intellectual enthusiasm for the obvious benefits which the altruistic suicide of damaged or excessive cells presented for multicellular organisms. Considering the problems involved in, for example, mammalian differentiation or immune defence, an elaborate system for the harmless disposal of unwanted cells is essential. As a result of extensive investigations of cell death pathways in metazoa, apoptosis is now widely accepted as a universal component of developmental and differentiation programmes. Active cell death is pivotal in multicellular organisms for maintenance of stable cell numbers, to engineer specialist cellular selection and control cellular functions in critical systems such as the immune, haemopoietic and nervous systems. Any failure of such cell control systems in mammals may lead to oncogenesis (Fadeel and Orrenius, 2005) or, within the immune system, to the development of autoimmune diseases (Williams, 1994).

There are two principal recognised means of cell death: the first, necrosis, describes any form of killing of a cell by a major damage, incompatible with life; causes may be injury, infection, cancer, infarction or inflammation, which eventually lead to a release of lysosomal enzymes as the final cause of death. As an early event, the integrity of the plasma membrane is damaged, leading to the uptake of otherwise impermeable dyes which is indicative of a necrotic cell and a marker for classification. The second, by contrast, is an active process know as programmed cell death (PCD) in which, after receiving an internal or external signal, the cell uses specialized cellular machinery to commit suicide. Several forms of PCD have been described: apoptosis, apoptosis-like PCD, paraptosis, autophagy, necrosis-like autophagy, all of which differ substantially in the molecular mechanisms of the induced death phenotype. The most investigated form of PCD is apoptosis and this process, first defined by Kerr *et al*. (1972), is characterized by typical morphological features including plasma membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation. Apoptosis is a tightly regulated and highly efficient programme for cell death which requires the interplay of a multitude of factors. It is not just a degradation process but is energy dependent and needs functional protein biosynthesis. The components of the apoptotic signalling network are genetically encoded and probably in place in any nucleated cell of a multicellular organism from *Caenorhabditis elegans* to man. Although the volitional doom of live cells seems somewhat teleological, a driving force during evolution is obviously a profitable outcome for the survival of the whole organism.

* Corresponding author: Sue Welburn. Email: sue.welburn@ed.ac.uk

PCD IN HOST-PATHOGEN RELATIONSHIPS: PREVENTION OR PROMOTION OF PARASITISM?

The term programmed cell death (PCD) is often used as a synonym for apoptosis (Lockshin and Williams, 1964). In eukaryotes, PCD is vital for the survival of multicellular organisms and is responsible for the programmed elimination of cells during development, removal of cancerous cells and cells that have become damaged (e.g. by infection). PCD

Parasitology (2006), **132**, S7–S18.
doi:10.1017/S0031182006000825 Printed in the United Kingdom

Table 1. Comparison of apoptosis in metazoa and protozoa.

	Metazoa		
	Extrinsic pathway	Intrinsic pathway	Trypanosomatids
Inducer	Death receptor ligand (e.g. TNFα)	Health status of the cell (e.g. viral infection)	Cytokine of parasite origin (e.g. PGD_2), ROS, procyclin mediated receptor binding
Initiator	Caspase 8	Caspase 9	Not identified
Effector	Caspases 3 and 7	Caspases 3 and 7	ROS and cation homeostasis
Activation of	Proteases and nucleases (including other caspases)	Proteases and nucleases (including other caspases)	Proteases and nucleases (metacaspases?)
Observed hallmarks			
– necessity of protein biosynthesis	+	+	+
– condensation of chromatin	+	+	+
– segmentation of nuclei	+	+	+
– degradation of DNA	+	+	+
– apoptotic bodies or blebbing	+	+	+
– exposition of phosphatidylserine	+	+	+
– release of cytochrome C	−	+	+
– decrease of mitochondrial membrane potential (Ψ_m)	−	+	+
Outcome	Cell degradation without inflammation	Cell degradation without inflammation	Parasite degradation without host inflammation

is not strictly confined to physiological control mechanisms; in addition to induced cell death due to a signal molecule, cells can undergo PCD in response to damage, particularly DNA damage (Clarke *et al*. 1993; Kim *et al*. 2005). This 'suicide' response is clearly altruistic, ensuring the survival of an entire organism by eliminating damaged cells. It is becoming increasingly apparent that PCD forms a crucial component of host-pathogen relationships and that many pathogens elicit an apoptotic response in the host cell which may inhibit or promote persistence of the pathogen in the host. Apoptosis of host cells can be induced by intracellular bacteria (Zychlinsky *et al*. 1992; Khelef, Zychlinski and Guiso, 1993) and intracellular protozoans, for example *Trypanosoma cruzi*, selectively induce activation-dependent apoptosis in host-infected $CD4^+$ but not $CD8^+$ T cells (DosReis *et al*. 1995; Lopes *et al*. 1995). Many mammalian cells respond to viral infection by entering apoptosis (Shen and Shenk, 1995); if PCD occurs early enough in the host cell after infection, there is a substantial reduction in the release of viral particles and consequent spread of the infection – the 'pre-lytic halt hypothesis' (Martz and Howell, 1989).

Recently apoptosis has been observed in mosquito tissues in response to malaria infection, promoting transmission of parasites through the mosquito midgut epithelium (Al-Olayana, Williams and Hurd, 2002; Hurd and Carter, 2004) but also contributing a negative impact on vector fitness due to apoptosis of the follicular epithelium (Ahmed and Hurd, 2006). Other intracellular protozoan parasites inhibit the apoptotic programme of the host cell including, *Toxoplasma gondii*, *Trypanosoma cruzi*, *Leishmania* spp., *Theileria* spp., *Cryptosporidium parvum* and the microsporidian *Nosema algerae* (Heussler, Kuenzi and Rottenberg, 2001). For example, macrophages parasitized by the infective trypanosomatid *Leishmania donovani* are prevented from undergoing apoptosis (Moore and Matlashewski, 1994) and *Plasmodium berghei* sporozoites have been observed to prevent apoptosis in hepatocytes (Van der Sand *et al*. 2005).

It has recently been proposed that apoptosis of both host and *T. cruzi* may contribute to the silent spreading and persistence of the parasite and a means of avoidance of triggering of an exacerbated inflammatory response by the host (de Souza *et al*. 2003). Furthermore, apoptotic mimicry by *Leishmania* parasites in the mammalian host through exposure of surface phosphatidylserine (PS) is believed to contribute to the internalization of *L. amazonensis* amastigotes (Wanderly *et al*. 2006).

PCD IN SINGLE CELLED ORGANISMS

Much of the genetic analysis of PCD has been carried out on the nematode *C. elegans* (Ellis, Yuan and Horvitz, 1991) and the fruit fly *Drosphila* (White *et al*. 1994). However, the boundaries defining regulated cell death pathways as a strictly multicellular phenomenon were significantly altered with the observation of PCD in the slime mould *Dictyostelium*

https://doi.org/10.1017/S0031182006000825 Published online by Cambridge University Press

discoideum, an organism phylogenetically far removed from vertebrates with both single- and multicellular stages in its life cycle (Cornillon *et al*. 1994). Moreover, some features of PCD in *Dictyostelium* (e.g. retention of cell membrane integrity until a late stage in the death process) are similar to those found in vertebrates. It may be that 'the evolutionary step from single to multicellular organisms appears to have been taken many times' (Kaiser, 1986) and it seems likely that a common mechanism for cell death emerged prior to the evolution of multicellularity. This idea is further supported by the observation of macronuclear loss in the protozoan *Tetrahymena thermophila* by a process that is similar to the nuclear changes occurring in PCD through apoptosis in mammalian cells (Davis *et al*. 1992; Christensen *et al*. 1995, 1998).

Implicit in the specific association of PCD with multicellularity is the idea that single-cell organisms have no need of an orderly process of cell death; what would be the point in a single-celled organism carrying the complex machinery for arranging its own death without the need for altruistic behaviour? Indeed, it has been questioned if protozoa are able to undergo apoptosis at all, because – considering the principle of survival of the fittest – it seems rather a contradiction that a single cell organism may have evolved a programme to kill itself. However, this leads inevitably to the idea that, unless killed by outside forces (inducing 'necrotic death'), protozoa have achieved a state of immortality with no apparent limits to their proliferation apart from those imposed by substrate or external means like antibodies or toxins. Acceptance of this line of thought permeates much of parasitology. Over the last decade a large body of evidence has been built up showing that protozoa also possess the ability to undergo programmed cell death. A spate of publications described a form of controlled or regulated cell death in three members of the order Kinetoplastidia from most ancient phylogenic branches of unicellular eukaryotic lineages: *Trypanosoma cruzi* (Ameisen *et al*. 1995), *T. brucei rhodesiense* (Welburn *et al*. 1996), and *L. amazonensis* (Moreira *et al*. 1996; Lee *et al*. 2002). All three trypanosomatids studied displayed common ultrastructural and biochemical characteristics usually associated with the apoptotic process in metazoa, including the characteristic fragmentation of DNA into oligonucleosomal repeat-sized fragments, suggestive of the evolution of a generalized mechanism of programmed cell death in these parasites (see Table 1). PCD in these organisms followed a very similar pattern of events, sharing many hallmark phenotypic features common to metazoan cells undergoing apoptosis (Wyllie, Kerr and Curry, 1980). Soon it became apparent that other protozoa such as *Plasmodium* (Picot *et al*. 1997; Al-Olayan *et al*. 2002, Deponte and Becker, 2004), *Trichomonas foetus* (Mariante *et al*. 2003), the ciliate *Tetrahymena thermophila* (Christensen *et al*. 2001) the dinoflagellate *Peridinium* (Vardi *et al*. 1999) and more recently *Trypanosoma musculi* (Gugssa *et al*. 2005), can all undergo a process similar to PCD in higher eukaryotes. Cell death has also been recorded in yeast and genetic markers observed in response to oxidative stress (Madeo *et al*. 1999; Laun *et al*. 2001) and acetic acid (Ludovico *et al*. 2001, 2002). There is also a large body of evidence in the literature, both morphological and molecular, pointing towards the existence of bacterial PCD as a response to various stress stimuli and during the course of certain developmental processes. Several authors (Hochman, 1997; Lewis, 2000; Ameisen, 2002) have suggested that PCD in prokaryotic organisms, as in eukaryotes, may lead to the elimination of cells during developmental processes and the removal of damaged cells. Lewis (2000) suggests that bacterial autolysis after exposure to antibiotics may involve bacterial PCD to eliminate damaged cells. Furthermore, it is possible that the spontaneous lysis of a large fraction of a bacterial population (but not the entire population), often observed in stationary phase bacterial cultures, may also represent an example of PCD that occurs in response to poor nutrient conditions (Rice and Bayles, 2003). The fact that PCD has now been documented in such a variety of unicellular eukaryotes suggests that this process is probably conserved throughout single-cell organisms.

SOCIAL ORDERING AND REGULATION OF PARASITE POPULATION DENSITY

It has been suggested that metazoan organisms utilize PCD as a form of self-regulation and furthermore that apoptosis and PCD form a system of social control on cell populations (Raff, 1992). It has been generally assumed that parasite death, whether in the mammal or in the vector, plays no part in the development and course of a parasitic infection, except when caused by external necrotic stimuli (e.g. toxin or antibody challenge). So much effort in parasitological research has gone into examining ways in which parasites may be killed due to drugs, antibodies etc., but less attention has been directed at examining parasite growth limitation or self-regulation. Since trypanosome populations are largely clonal (Tibayrenc *et al*. 1991), it would be an advantage to possess an altruistic mechanism to promote and maintain genetic stability within the population. Uncontrolled growth would lead to death of the vector and or host with the consequence that none of the parasites of a clone would complete their life cycles. A uniform characteristic of the trypanosomatids in which PCD (or apoptotic-like processes) have been described is that they spend lengthy periods within their mammalian hosts and insect vectors.

There is no reason to suppose that PCD does not also play a role in the mammalian cycle of *T. brucei* and perhaps other protozoan parasites. The fluctuating parasitaemias, characteristic of protozoan infections within the mammalian bloodstream, may not only reflect the efforts of the mammalian immune system engaged in their destruction but also the parasites ability to regulate its cell density. In the mammalian host they cause waves of parasitaemias, due to the process of antigenic variation (Cross, 1996). After the appearance of a new antigenic variant the host will mount an antibody-mediated immune response which effectively clears the parasite population expressing that antigen. However, some parasites switch their coat, thereby escaping the immune response until the host mounts a response to the new antigenic coat. This process gives rise to the classical progression of a trypanosome infection first noted by Ross and Thomson (1910). This process has been simulated *in vitro* culture of trypanosomatids, whereby it is accepted that these organisms regulate their own numbers to suit the prevailing culture conditions; limiting the intensity of infection and total medium exchanges at regular time intervals similarly resulted in parasitic waves without the presence of any antibodies (Hesse *et al.* 1995). In culturing bloodstream form trypanosomes Hesse *et al.* (1995) found that higher parasite cell concentrations could only be obtained by total medium replacement; neither addition of fresh medium nor serum led to a higher cell yield, suggesting that a trypanosome-derived factor accumulated during cultivation and was involved in regulating the population size. Cell density has also been shown to impact on parasite death and differentiation *in vivo*; Vassella *et al.* (1997) showed *in vitro* that cell density dependent trypanosomes would transform to non-dividing stumpy forms and more recently it has been shown that prostaglandin D_2, which is produced principally by stumpy forms, is able to induce cell death of these forms, representing a second control point after the terminal differentiation to the stumpy form (Figarella *et al.* 2005). Likewise, the epimastigote form of *T. cruzi* underwent massive apoptotic cell death during progression to the G0/G1 arrested trypomastigote stage, accounting for the so called 'stationary phase' of the culture (Billaut-Mulot *et al.*, 1996). The fact that cell death could be accelerated or prevented by modifying culture conditions, strongly suggests that trypanosomes use extracellular signals to regulate their survival, differentiation and even PCD.

Similarly, in the mammal it is well established that individual cells in many protozoan populations are not always proliferating at the highest possible rate in order to outgrow each other. Rather, individual cell proliferation is modulated by host-derived signalling molecules, growth factors and cytokines (Barcinski and Moreira, 1994). In the interactions among host cells, *Leishmania* parasites and cytokines, a glycoprotein antigen from promastigote *L. amazonensis* (Rodrigues *et al.* 1986) induced production of a lesion enhancer molecule in T cells which also acts as a haemopoetic growth factor (Rodrigues *et al.* 1987). Supernatant from these cultured T cells promoted the multiplication of promastigotes *in vitro*, attributed to granulocyte macrophage colony stimulating factor (GMCSF) (Charlab *et al.* 1990). Since the vertebrate host interacts only with non-dividing promastigotes, the physiological relevance of this result was unclear. However, when the growth temperature of *L. amazonensis* promastigotes was increased from that of the sandfly vector (22–28 °C) to that of the mammalian host (34–37 °C), parasites underwent calcium-dependent PCD (Moreira *et al.* 1996). Since GMCSF has been shown to protect infective promatigotes from heat induced death *in vitro* (Barcinski *et al.* 1992) and infection with *L. amazonensis* (Soares and Barcinski, 1992) induces macrophage production of GMCSF, parasites may be protected from death by this cytokine at the onset of a mammalian infection.

In the insect vector, the parasites must control the population of vector-infective forms and in some cases select individuals within the core population for progression to mammalian infective forms. Even successful maturation is preceded by a long incubation period in the gut when parasites are in direct competition with the vector for limited sources of proline as an energy source. Analysis of trypanosome numbers in infected tsetse midguts between 9 and 26 days post infection showed that the midgut population was maintained at a remarkably constant level, both within and between flies, strongly suggestive of a mechanism of self-regulation of the protozoan population within the vector (Welburn and Maudlin, 1997).

ALTRUISM AND THE MAINTENANCE OF GENETIC STABILITY

When cell populations are clonal or oligoclonal, the self-destruction or terminal differentiation of individual cells can be justified by the perpetuation of the life cycle by other closely genetically related cells. For example, in the mammalian immune system apoptosis is necessary for the control of cell populations at the clonal level. We are becoming increasingly aware that single cell organisms display social behaviour. Bacteria, for example, co-ordinate gene expression and group behaviour by quorum sensing. Rice and Bayles (2003) suggest that bacteria altruistically self-sacrifice the majority of the population, after exposure to a damaging agent such as antibiotics, to enhance the survival of the few that remain. Such self-sacrifice of a damaged single cell or sub-population of cells would benefit the undamaged

clonal relatives by sparing nutrient resources and therefore ensure survival of the population's genome (Froehlich and Madeo, 2000).

The strategy adopted by trypanosomatids appears to be a self-perpetuating and interdependent system, with specific attributes and tasks distributed within different sub-populations. Trypanosomes live in populations that are permanently under selection; advantageous phenotypes will be promoted and the disadvantageous (e.g. those which cannot mature to become infective to humans and complete the life cycle) may have to be removed. Parasites of *Trypanosoma brucei* subspecies in particular appear to follow designated pathways during their life cycles; some trypanosomes in the mammal undergo 'terminal differentiation', whereby some cells differentiate from dividing slender to non-dividing stumpy forms, while the remaining population continues to divide. Over the last few years, however, it has become apparent that bloodstream form trypanosomes can also undergo programmed cell death. It has been hypothesised that stumpy bloodstream form *T. brucei* may be heterogeneous, made up of a population of newly formed transformed cells which are capable of completing differentiation in the insect vector and a population of older cells that have entered an apoptotic state which is no longer able to survive (Seed and Wench, 2003). The possibility that stumpy cells may undergo apoptosis was first reported by Welburn and Murphy (1998) as they showed up-regulation of TRACK, a gene also up-regulated in dying procyclic forms treated with concanavalin A. Stumpy form trypanosomes are unable to switch their VSG coat and will be removed by the host antibody response thereby reducing the parasitaemic burden. Experiments in laboratory rodents have shown that monomorphic laboratory strains of trypanosomes (which show reduced ability to change to the short stumpy form) are more pathogenic than pleomorphic strains from the field, suggestive of evolutionary advantage of pleomophology to limit the parasite's population density and pathogenicity (Black, Jack and Marrison, 1983). Since trypanosome are largely clonal, altruistic behaviour, which permits clones expressing a switched VSG coat a greater chance of being picked up by the tsetse, may provide an evolutionary advantage. PCD induction in the bloodstream form, specifically in the stumpy form, may play a major role in the regulation of cell density within the host. Elimination of stumpy parasites would not only diminish the parasitaemia by elimination of the non-dividing stumpy form, but would also not compromise the infection, because the slender form (dividing stage) will be not affected by this process.

Likewise, only a proportion of procyclic parasites in a persistently infected tsetse gut are destined to differentiate into mature mammalian infective and complete the life cycle. The process of parasite maturation to mammalian infective forms (metacyclic) whereby some parasites migrate from the midgut to the salivary glands (in the case of *T. brucei*) or proboscis (in the case of *T. congolense*), is by no means a foregone conclusion and many established (procyclic) infections never mature as evidenced by low salivary gland infection rates seen in the wild (Okoth and Kaapata, 1986) and in the laboratory (Welburn, Maudlin and Milligan, 1995).

Similarly, in *Leishmania* during the digenetic cycle, some individuals may be programmed to infect and suffer 'terminal differentiation', while others are ancillary to the former and still others are present to maintain a certain population density for infection (Moiera *et al*. 1996).

CELL DEATH AND OXIDATIVE STRESS IN INSECT FORM *TRYPANOSOMA BRUCEI*

The majority of bloodstream form parasites taken in by the tsetse with an infective meal fail to successfully establish a persistent infection in the ectoperitropic space of the fly. This failure is not a consequence of an intrinsic inability of bloodstream forms to transform to the procyclic form (dying forms observed in the tsetse fly midgut have already successfully transformed to the insect form) but is due to the fact that post transformation the parasites undergo cell death (Welburn, 1989). These dying parasites in the insect gut display characteristic apoptotic morphology, including surface membrane vesiculation and condensation of chromatin at the periphery of the nuclear membrane, while mitochondria remain intact (Welburn, Maudlin and Ellis, 1989). This process of cellular death is not unique to *T. brucei*, since *T. vivax* and *T. congolense* also undergo cell death in this manner eliminating the possibility that the short stumpy/long slender hypothesis is involved in the process (there are no such forms reported in *Nannomonas* spp.). An analogous phenomenon can be observed *in vitro*, when parasites are treated with the plant lectin concanavalin A (ConA) whereby cell death culminated in cleavage of nuclear DNA into oligonucleosomal fragments late in the cell death process (Welburn *et al*. 1996). The receptor for induction of Con A-induced cell death is the insect form major cell surface glycoprotein, procyclin (Pearson *et al*. 2000). The cell death process is associated with differential expression of mRNAs (Murphy and Welburn, 1997) including prohibitin and TRACK, a receptor for protein kinase C (Welburn and Murphy, 1998), a QM homologue (Lillico *et al*. 2002) and MOB 1 (Hammarton *et al*. 2005). Mitochondrial involvement in ConA induced cell death is implied by the identification of a cDNA with homology to a mitochondrial RNA splicing protein, a mitochondrial transporter and cytochrome c1 (Welburn, Lillico and Murphy, 1999).

https://doi.org/10.1017/S0031182006000825 Published online by Cambridge University Press

It has recently been shown that trypanosomes taken in with an infective blood meal are normally killed in the midgut by reactive oxygen species (ROS) produced during the digestion of the blood meal (MacLeod, 2005). Blood is the sole diet of both sexes of tsetse flies and breakdown of the bloodmeal promotes oxidative stress in the midgut, with haemin, iron and haemoglobin all driving Fenton reactions (Souza *et al*. 1997). The addition of antioxidants to the tsetse blood meal was shown to significantly increase trypanosome midgut infection rates in *G. m. morsitans*, suggesting that reactive oxygen species play a major role in killing trypanosomes entering the fly midgut. For example, addition of glutathione (GSH), the main cellular antioxidant, could raise trypanosome infection rates to 100% in *G. m. morsitans*. Trypanosomes, however, use trypanothione as their main cellular antioxidant (Fairlamb *et al*. 1985). The increased susceptibility was shown to be independent of protein synthesis by use of the non-physiological isomer D-cysteine (which gave identical results to L-cysteine), suggesting that antioxidants act directly on the midgut environment. N-acetyl-cysteine (NAC) which has the ability to cross cell membranes (Abello, Fidlerm and Buchman, 1994; Laragione *et al*. 2003) was also found to enhance trypanosome infection rates but 10 fold less NAC concentration was required to promote ~100% infection than was required for GSH or cysteine, suggesting that protection of the intracellular environment from oxidative stress is similarly important for the trypanosome survival. This suggests that either oxidative molecules produced in the midgut environment penetrate the trypanosome membrane, disrupting the internal homeostasis of the parasite or that during parasite transformation from bloodstream to procyclic form, oxidative stress is induced in the trypanosome. Ridgley, Xiong and Ruben (1999) had shown that oxidative stress (through addition of a hydrogen peroxide producing enzyme) could induce apoptosis in procyclic form trypanosomes with characteristic DNA fragmentation.

CELL DEATH AND OXIDATIVE STRESS IN BLOODSTREAM FORM *T. BRUCEI*

Cultured at high density, bloodstream forms show apoptotic characteristics (Tsuda *et al*. 2005) which, during natural infections, may also help to regulate parasite numbers in the mammalian host. However, when the incubation temperature was dropped from 37 °C to 27 °C, the trypanosomes developed increased resistance to apoptosis. This resistance was associated with an increase in expression of the enzyme trypanosome alternative oxidase (TAO). TAO is the sole terminal oxidase in bloodstream forms and is responsible for removing excess reducing equivalents produced during glycolysis in the GAP DH reaction by transferring oxygen to water (Chaudhuri, Ajayi and Hill, 1998; Fang and Beattie, 2003). Inhibition of TAO (by the addition of ascofuranone to the culture medium) was shown to increase levels of PCD in bloodstream form trypanosomes cultured at 27 °C suggestive of TAO inhibiting PCD (Tsuda *et al*. 2005). Fang and Beattie (2003) had previously shown that inhibition of TAO resulted in increased production of ROS, while Ridgley *et al*. (1999) demonstrated that ROS could induce cell death in the procyclic form of *T. brucei*. It is thus not surprising that the increased production of ROS by inhibition of TAO could induce programmed cell death in bloodstream forms.

PCD has recently been shown to occur in bloodstream forms cultured with prostaglandin D_2 (Figarella *et al*. 2005). Trypanosomes produce prostaglandins (especially PGD_2, which *in vitro* is primarily secreted into the medium; Kubata *et al*. 2000), which most likely play a significant role in the host-parasite relationship, as many of the symptoms of sleeping sickness are related to known effects of PGD_2 on mammals, such as pain, fever immunosuppression or sleep induction. Secreted PGD_2 may be one of the trypanosome derived factors observed in culture experiments (Hesse *et al*. 1995). Treatment of bloodstream forms with PGD_2 (but not with PGE_2 and $PGF_{2\alpha}$) led to an apoptosis-like PCD phenotype, which could be blocked by pre-treatment with protein synthesis inhibitors. The effect of ROS on PCD in bloodstream forms has also been investigated (Figarella *et al*. 2006). Bloodstream form trypanosomes have previously been show to undergo PCD in the presence of PGD_2 (Figarella *et al*. 2005) and recently PGD_2 metabolites of the J_2-series have also been shown to induce PCD. When these prostaglandins were added to cultured bloodstream trypanosome forms an increase of intracellular ROS was observed prior to the appearance of any characteristics of PCD. The ability to generate ROS was unique for each prostanoid and was closely correlated with their capacity to cause PCD. Intracellular ROS production induced by these prostaglandins in the bloodstream form was completely abolished by pre-treatment with NAC or glutathione, which led to efficiently reduced signs of PCD. The effect was more potent in parasites in stationary phase or parasites treated with cAMP analogues, which were shown to induce differentiation from slender to stumpy form (Breidbach, Ngazoa and Steverding, 2002). These data suggest that PGD_2 induces PDC primarily in stumpy bloodstream forms. The PCD phenotype induced by PGD_2 was not modified in any way by the caspase inhibitors zVAD and DEVD-CHO, suggesting that the process is caspase independent. Likewise, we have recently been able to show that prostaglandin-induced cell death depends on oxidative stress, since use of ROS scavengers rescued parasites from PCD (Figarella *et al*. 2006). In contrast, incubation of parasites with prostaglandin

https://doi.org/10.1017/S0031182006000825 Published online by Cambridge University Press

and SHAM (a TAO inhibitor) accelerated cell death (Figarella, 2005).

Taken together, these data suggest that ROS play an important role as intermediates in the PCD signalling pathways in both procyclic and bloodstream form *Trypanosoma brucei*. Oxidative stress and the REDOX state of the cell is one of the main factors controlling apoptosis in multicellular organisms (Curtin, Donovan and Cotter, 2002; Le Bras *et al*. 2005; Li and Wogan, 2005). Oxidative stress-mediated cell death has been implicated in the aetiology of numerous common diseases including cancer, neurodegenerative disorders and heart failure (Engel and Evens, 2006; Chong, Li and Maiese, 2005; Naoi *et al*. 2005; Andreka *et al*. 2004). Free radicals have been shown to limit the development of a range of parasites in their invertebrate hosts (Ascenzi and Gradoni, 2002). Exposure of *Leishmania amastigotes* to moderate concentrations of NO-donating compounds or to endogenous NO produced by lipopolysaccharide or gamma interferon treatment of infected macrophages resulted in a dramatic time-dependent cell death in these parasites (Holtzmuller *et al*. 2002). However, NO which has been shown to limit development of malarial parasites in the mosquito *Anopheles stephensi* (Luckhart *et al*. 1998) appears to play no significant role in preventing the establishment of midgut infections in tsetse (Hao, Kasumba and Aksoy, 2003).

EFFECTOR MECHANISMS IN TRYPANOSOMES

In mammalian cells, apoptotic cell death is classically considered to be carried out by a class of cysteine proteinases called caspases. They exist within the cell in an inactive state until activated by limited proteolysis by a variety of extracellular or intracellular effector molecules (Bialik and Kimchi, 2006). It was also shown that the NO-mediated modification of the catalytic centre of cysteine proteases, including those from Coxsackievirus, Rhinovirus and *Leishmania infantum*, as well as falcipain, papain, calpain, the cathepsins and mammalian caspases, blocks the enzyme activity *in vitro* and *in vivo* (Ascenzi *et al*. 2001). There is now accumulating evidence indicating that cell death can occur in a programmed fashion but in the complete absence of and independent of caspase activation. Caspase-independent cell death pathways are important safeguard mechanisms to protect the organism against unwanted and potentially harmful cells when caspase-mediated routes fail but can also be triggered in response to cytotoxic agents or other death stimuli. As in apoptosis, the mitochondrion can play a key role but also other organelles such as lysosomes and the endoplasmic reticulum have an important function in the release and activation of death factors such as cathepsins, calpains, and other proteases (Broker, Kruyt and Giaccone, 2005).

Although there is no indication that trypanosomes (or other protozoa) possess classical apoptotic caspases, as judged from their genome analyses, there are some reports, which describe caspase-like activity in the protozoa *Leishmania* (Sen *et al*. 2004), *Tetrahymena* (Kobayashi and Endoh, 2003) and *Blastocystis* (Nasirudeen *et al*. 2001; Tan and Nasirudeen, 2005). Although cysteine proteases are typical lysosomal enzymes, they are actually recognized as multi-function enzymes, being involved in antigen processing and presentation, in membrane-bound protein cleavage, as well as in degradation of the cellular matrix and in processes of tissue remodelling.

Campothecin-induced PCD in *L. donovani* is characterized by an increase in ROS inside cells, which causes subsequent elevation in the level of lipid peroxidation and decrease in reducing equivalents. Endogenous ROS formation and imbalance in intracellular cation homeostasis led to a reduced mitochondrial membrane potential and cytochrome c release into the cytosol. These events were followed by activation of caspase-like proteases and finally induced PCD in *Leishmania* (Sen *et al*. 2004). In *T. cruzi* epimastigotes, PCD could however be inhibited by L-arginine by means of a NOS-dependent NO production, suggesting again that in these parasites apoptosis may depend on a caspase-like protease (Placenza, Peluffo and Radi, 2001).

Several cysteine proteases have been described in *T. brucei* which play an important role in normal function as their inhibition results in parasite death (Scory *et al*. 1999; Troeberg *et al*. 1999). For example, the activity of the cysteine protease rhodesain in *T. brucei* has been observed to be higher in stumpy than in slender bloodstream form parasites or procyclics (Caffrey *et al*. 2001); however, rhodesain is found in the lysososome and therefore its main function seems to be the degradation of proteins from both parasite and host origin.

Although apoptosis has been initiated in *S. cerevisiae* by expression of Arabidopsis metacaspases (Watanabe and Lam, 2005) and *T. brucei* metacaspases IV resulted in growth inhibition, mitochondrial dysfunction and clonal death when expressed in *S. cerevisiae* (Szallies, Kubata and Duszenko, 2002) to date, no demonstrable protease activities for the trypanosomal metacaspases have been observed (Zannager, Mottram and Fasel, 2002).

FUTURE PERSPECTIVES

Considering the experimental evidence, there can be little doubt that unicellular organisms and especially the parasitic protozoa do undertake PCD. These parasites face the constant risk of an uncontrolled super-infection which would lead to the premature death of the host and a reduced chance of continuing with their life-cycle. Removal of part of the

population, flagged by cell-density as described above, is an altruistic form of PCD and the counterpart of apoptosis in metazoa. The molecular mechanisms defining the architecture of apoptosis in protozoa however remain to be resolved. Metacaspases, as the protozoan paralogues of caspases do not seem to play a pivotal role in the initiation of apoptosis and may only be involved in the process (together with other cysteine proteases and nucleases) as tools for the necessary degradation reactions. The initiation of protozoan apoptosis is associated with the formation of ROS and cation (especially potassium and calcium) homeostasis seems particularly critical to trypanosomes. ROS are probably formed within the mitochondrion and this may provide the link to metazoan apoptosis. Indeed, protozoa may exhibit a rather primitive form of apoptosis which during evolution has led to the specialization of metacaspases to become caspases as defined and controlled check points of cell death. In this way, death receptor dependent apoptosis could be viewed as an acquired second pathway specifically developed in metazoa.

Future work should concentrate on the chronology of protozoan apoptosis, defining the major players in and the genetic basis of this form of cell death. The important intracellular compartments have to be localized and the role of proteases and nucleases elucidated. Research should also explore protozoan apoptosis for its progenitor function of apoptosis in higher eukaryotes. Most importantly only artificial inducers like stress conditions, camptothecin, staurosporin, externally added NO and others have to date been used to induce PCD in protozoa. What is needed is information about the physiological inducer(s). Prostaglandin D_2 may provide one such candidate since it is synthesized and secreted by stumpy form trypanosomes and may itself work as a cytokine. However, we have still not identified the receptor molecule in trypanosomes – we have no indication for a classical membrane bound PG receptor and a homologue of PPARγ remains elusive although troglitazone leads to parasite differentiation (Denninger and Duszenko, unpublished observations). A detailed analysis is needed of the first-line effector molecules, of which ROS is a candidate; the chemical nature of this and other molecules at this position of the event cascade should be identified and used to explore the progression of cell death.

Leaving aside our natural desire to identify a paradigmatic apoptosis in protozoa, from which the evolution of apoptosis can be reconstructed, the understanding of apoptosis in protozoan parasites offers a substantive additional reward. Trypanosomes (and kinetoplastids more generally) are unable to survive independently of a complex cellular (host or vector) environment. From our own selfish point of view, the altruism of the protozoa could represent their weakness. The biochemical events leading to cell death, in for example both mammalian and insect form *T. brucei*, may provide key novel therapeutic targets for these pathogens that could be exploited in the design of specific and non-, or at least less toxic, chemotherapeutic agents.

ACKNOWLEDGEMENTS

This work was supported by the Wellcome Trust (SCW) and the Deutsche Forschungsgemeinschaft (M.D.). K.F. is a recipient of a DAAD Scholarship.

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https://doi.org/10.1017/S0031182006000825 Published online by Cambridge University Press