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Cell Death and Human Intestinal Protozoa: A Brief Overview

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Abstract

Protozoan programmed cell death or apoptosis is an important factor in the survival of the parasite and its pathogenicity. The most amazing aspect of protozoan cell death is in its molecular architecture. To date, protozoa lack most of the components of the highly complex cell death machinery studied in multicellular organisms. Hence the unique apoptotic machinery in protozoa can be exploited for the development of therapeutic drugs and diagnostic markers. This review focuses on human intestinal protozoa undergoing cell death and inducing or inhibiting host cell apoptosis. The first part of this review focuses on intestinal protozoa that undergo PCD under various stress conditions. The second part focuses on protozoa that induce or inhibit PCD in their host cell. Although these intestinal parasites differ in their mechanism of infection and intracellular localization, they may activate conserved cell death pathways within themselves and in the host cell. Understanding conserved cell death pathways in the intestinal protozoa and their host-parasite PCD relationship may lead to drug targets which can be used for a broad range of parasitic diseases.

Introduction

Programmed cell death has been noted in all invertebrate and vertebrate multicellular organisms studied so far, including nematodes, insects, amphibians and mammals (Ellis *et al.*, 1991; Raff, 1992; Vaux, 1993; Steller, 1995). Programmed cell death or apoptosis is associated with characteristic morphological and biochemical changes, including cell shrinkage, membrane blebbing, cell surface changes and loss of mitochondrial function (Heusler *et al.*, 2001). These cell membrane alterations include exposure of phosphatidylserine residues on the outer leaflet of the plasma membrane (McConkey *et al.*, 1996). Apoptosis also displays characteristic nuclear changes. The chromatin undergoes condensation as endonucleases are activated and the DNA begins to fragment (Heusler *et al.*, 2001).

There are three main reasons why cells commit suicide. Firstly, PCD is needed for proper development of an organism, for example the resorption of the tadpole tail at the time of its metamorphosis into a frog. Secondly, PCD is needed to destroy cells that represent a threat to the integrity of the organism, for example virus-infected

cells (Vaux *et al.*, 1994). Thirdly, cells dying by PCD do not cause inflammation of the surrounding cells as the cellular contents are packaged as apoptotic bodies and the dying cells are taken up by macrophages or neighbouring cells (Kerr and Harmon, 1991).

It is now clearly evident that, in higher vertebrates, PCD is important for the physiological development of the organism. Hence it may be assumed that PCD only serves multicellularity and there is no possible reason to assume that unicellular organisms could benefit from such a cell death mechanism. Death of a cell or a group of cells in multicellular organisms may lead to the development or survival of other cells in the organism. However, death of a cell in an unicellular organism would mean the death of the entire organism (Vaux and Strasser, 1996; Barcinski and DosReis, 1999). Single-celled organisms are also not thought to age the way plants and animals with predetermined lifespan do (Vaux and Strasser, 1996), although asymmetrically dividing yeasts have been observed to die after giving rise to a certain number of daughters (Kennedy *et al.*, 1995).

Recent evidences, however, show that unicellular organisms can organize themselves as cell populations and establish patterns of intercellular communication (Shapiro, 1995; Palkova *et al.*, 1997) and some can respond to growth factors and cytokines that are otherwise regulators of PCD (Barcinski and Moreira, 1994). Among unicellular eukaryotes, PCD can be used as a defense mechanism for preservation of cell populations in times of viral infections, nutrient insufficiency and other adverse conditions, ensuring that a few cells survive to propagate the genome (Engelberg-Kulka and Glaser, 1999; Fröhlich and Madeo, 2000; Jin and Reed, 2002). Morphological alterations in unicellular eukaryotes undergoing PCD or apoptosis closely resemble apoptosis in mammalian or multicellular organisms. These include chromatin condensation, nuclear fragmentation, intact membranes and blebbing (Ameisen *et al.*, 1995; Welburn *et al.*, 1996; Murphy and Welburn, 1997). Some protozoan parasites directly regulate host cell apoptosis under certain conditions to ensure their survival within the host (Barcinski and DosReis, 1999). Among the many human intestinal protozoa, PCD, either in the parasite or the host cell, has only been investigated in a number of protozoa, namely *Blastocystis hominis*, *Cryptosporidium parvum*, *Giardia lamblia* and *Entamoeba histolytica*.

PCD in intestinal protozoa

PCD or apoptosis in protozoa has only been investigated in two intestinal parasites: *Blastocystis hominis* and *Giardia lamblia*.

Blastocystis hominis

Blastocystis hominis is an unicellular eukaryotic protozoan parasite found in humans and many animals (Zierdt, 1991;

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Boreham and Stenzel, 1993). *Blastocystis hominis* is often the most frequent protozoan found in the fecal samples of both patients suffering from intestinal disorders (Pikula, 1987; Zierdt *et al.*, 1995) and healthy individuals (Walker *et al.*, 1985; Ashford and Atkinson, 1992).

It is currently unknown if *B. hominis* is capable of causing disease. Whether it is a true pathogen or a commensal or is capable of being a pathogen in specific circumstances is currently a matter of debate (Stenzel and Boreham, 1996). Numerous clinical and epidemiological studies either implicate or exonerate the parasite as a cause of intestinal disease. There have been many reports suggesting that *B. hominis* causes disease (Taylor *et al.*, 1985; Sheehan *et al.*, 1986; Russo *et al.*, 1988) and there also have been a number of reports to the contrary (Sun *et al.*, 1989; Senay and MacPherson, 1990; Rosenblatt, 1991; Udkow and Markell, 1993). Although edema and inflammation of the intestinal mucosa have been noted in *B. hominis* infections (Lee *et al.*, 1990; Garavelli *et al.*, 1991; Russo *et al.*, 1988; Zuckerman *et al.*, 1994), endoscopy and biopsy results have indicated that *B. hominis* does not invade the colonic mucosa in human patients (Kain *et al.*, 1987; Tsang *et al.*, 1989; Dawes *et al.*, 1990; Zuckerman *et al.*, 1990). In contrast, another study described colonic ulceration with *B. hominis* present in superficial ulcers and infiltrating to the superficial lamina propria and gland spaces (Al-Tawil *et al.*, 1994).

There is growing evidence to suggest that immunocompromised individuals are more likely to suffer *Blastocystis*-related diarrheal illness (Prasad *et al.*, 2000; Tasova *et al.*, 2000). In immunocompromised patients, particularly patients with AIDS, *B. hominis* is suggested to be an opportunistic pathogen (Narkewicz *et al.*, 1989; Rolston *et al.*, 1989). Patients with AIDS were said to be more likely to carry *B. hominis* than those in the early stages of HIV infection (Albrecht *et al.*, 1995). In immunocompetent individuals, *B. hominis* infections cause intestinal disorders such as diarrhea, abdominal discomfort, anorexia, flatulence and irritable bowel syndrome (Boreham and Stenzel, 1993; Hussain *et al.*, 1997; Giacometti *et al.*, 1999).

We investigated the existence of a PCD machinery in this organism. An isolate of *B. hominis* (isolate B) succumbed to a cytotoxic monoclonal antibody (1D5) with a number of cellular and biochemical features characteristic of apoptosis in higher eukaryotes. Monoclonal antibody 1D5 bound preferentially to a 30.5 kDa plasma membrane-associated antigen and caused programmed cell death (PCD). In another study, apoptosis-like features were also observed in growing cultures of axenic *B. hominis* to metronidazole, a drug commonly used to treat blastocystosis.

Cell shrinkage due to compaction of organelles in the cytoplasm is an important morphological indication of apoptosis. This could be due to loss of cytoplasmic fluids and denaturation of proteins in apoptotic cells (Huppertz *et al.*, 1999). Light microscopy and flow cytometry revealed mAb 1D5- and metronidazole-treated *B. hominis* with condensed cytoplasm and darkening of cells (Nasirudeen *et al.*, 2001a, 2004a). Transmission electron micrographs of mAb 1D5- and metronidazole-treated *B. hominis* clearly revealed ultrastructural characteristics of apoptosis.

Cell shrinkage, nuclear condensation, deposition of membrane-bound apoptotic bodies, maintenance of cytosolic organelle structure and size and heavy vacuolization clearly provide ultrastructural evidence of apoptotic-like cell death in *B. hominis* (Nasirudeen *et al.*, 2001a). Externalization of phosphatidylserine residues, *in situ* DNA fragmentation and loss of mitochondrial membrane potential were also noted in *B. hominis* PCD (Nasirudeen *et al.*, 2001a, 2004a).

Enzyme-linked immunosorbent assay (ELISA) and immunoblot assay suggested that caspase-3-like antigens exist in *B. hominis*. Using colorimetric and flow cytometric assays for caspase-3-like activity, we also observed an increase in caspase-3-like protease activity in apoptotic *B. hominis* (Nasirudeen *et al.*, 2001b). Reverse transcriptase PCR resolved several distinct fragments on agarose gel electrophoresis. Upon sequencing, no homology to mammalian caspase-3 gene was noted. To date, no homologues of mammalian apoptotic genes have been reported in protozoa.

Giardia lamblia

Giardia lamblia is also capable of undergoing programmed cell death with features resembling apoptosis via a caspase-dependent pathway (Chose *et al.*, 2003). *Giardia lamblia*, the etiological agent of giardiasis, is the most commonly identified cause of water-borne disease (Marshall *et al.*, 1997). *Giardia lamblia* infection is characterized by acute or chronic diarrhea, dehydration, abdominal discomfort and weight loss (Farthing, 1996). Chose *et al.* (2003) reported that when treated with staurosporine or etoposide, *G. lamblia* undergoes apoptotic-like cell death displaying nuclear fragmentation, chromatin condensation, cytoplasmic vacuolation and formation of apoptotic bodies. The authors hypothesize the involvement of a caspase-dependent pathway but have yet to show any data using caspase inhibition assays. Internucleosomal fragmentation or *in situ* DNA fragmentation (using TUNEL) was also not detected in *G. lamblia* PCD (Chose *et al.*, 2003).

Protozoa-regulated PCD in host cell

Protozoa benefit in several ways from the host cell. The cell continuously supplies nutrients that are essential for parasite growth and may shield the intracellular parasite from the host immune system. Therefore, it is vital for the parasite to regulate PCD of the host cell.

Cryptosporidium parvum

Cryptosporidium parvum is an intracellular protozoon which causes persistent diarrhea and malnutrition in children (Ojcius *et al.*, 1999). The severe diarrhea is self-limiting in immunocompetent people and in immunocompromised patients infection can lead to serious complications including fatality. The primary site of infection of *C. parvum* is the epithelium of the intestine (McCole *et al.*, 2000).

Infection of intestinal and biliary epithelial cell lines with *C. parvum* results in host cell apoptosis leading to apoptotic nuclear condensation and DNA fragmentation in host cells (Ojcius *et al.*, 1999). But these apoptotic characteristics, nuclear condensation and DNA

fragmentation, were prevented by a caspase inhibitor (Ojcius *et al.*, 1999). It was also shown that blocking apoptosis with the caspase inhibitor increased the percentage of infected cells. These results suggested that *C. parvum*-induced apoptosis is caspase-dependent and that the parasite may use apoptosis to exit from the infected cell or that the infected cells may eliminate the parasite through apoptosis (Ojcius *et al.*, 1999).

Cryptosporidium parvum was reported to induce apoptosis in biliary epithelia via a Fas/Fas ligand-dependent mechanism and the parasite is capable of inhibiting host apoptosis by activating nuclear factor- κ B (NF- κ B) (Chen *et al.*, 1999, 2001). It was also reported that apoptosis in *C. parvum*-infected cells increases when NF- κ B is inhibited (McCole *et al.*, 2000). Using neonatal mouse models, Sasahara *et al.* (2003) showed apoptosis of the intestinal crypt epithelium in *C. parvum*-infected hosts. These reports suggest that apoptosis may play a significant role in the pathogenesis of cryptosporidiosis and that therapeutic interference with host apoptosis could be a means to treat the infection.

Mele *et al.* (2004) demonstrated that *C. parvum* is able to promote or inhibit apoptosis in the host cell depending on its developmental stages. At the trophozoite stages, *C. parvum* inhibits host cell apoptosis since the parasite needs the host cell for its own survival. At the sporozoite and merozoite stages, *C. parvum* promotes host cell apoptosis.

Giardia lamblia

Giardia trophozoites infect and colonize the proximal small intestine and adhere to the apical surface of the enterocyte leading to apoptosis (Farthing, 1996). Not all strains, however, cause apoptosis in enterocytes. Chin *et al.* (2002) demonstrated a strain-dependent induction of enterocyte apoptosis contributing to the pathogenesis of giardiasis. *Giardia lamblia* strains NF and S2 (but not strains WB or PB) induced apoptosis in enterocytes. *Giardia lamblia*-induced apoptosis in enterocytes was inhibited by treatment with the caspase-3 inhibitor Z-DEVD.FMK, suggesting the role of caspases in *G. lamblia* pathogenesis. *Giardia lamblia*-induced host cell apoptosis was characterized by the presence of cytoplasmic vacuolation, chromatin condensation, DNA fragmentation and nuclear membrane delamination (Chin *et al.*, 2002).

Entamoeba histolytica

Entamoeba histolytica is the major cause of amoebic dysentery and liver abscesses (Stanley, 2003). *Entamoeba histolytica* triggers apoptosis in neutrophils. Neutrophils incubated with *E. histolytica* showed characteristics of apoptosis: compaction of nuclear chromatin and swelling of nuclear envelope (Sim *et al.*, 2004). *Entamoeba histolytica*-induced Jurkat leukemia T cell apoptosis occurs through a caspase-dependent mechanism (Huston *et al.*, 2000). Huston *et al.* (2003) hypothesized that apoptotic killing and phagocytosis of Jurkat T cells by *E. histolytica* limits the leakage of toxic intracellular contents of the killed cells. *Entamoeba histolytica*-induced apoptosis and phagocytosis of Jurkat leukemia T cells limit inflammation and enable the parasite to escape the host immune system.

Perspectives

Vaux and Strasser (1996) observed that many drugs, toxins and physical insults provoke the same apoptotic response. They commented that it would be wrong to conclude that drugs and toxins act primarily to cause apoptosis; rather, the cell responds to insults by activating the apoptotic mechanism. In the case of *B. hominis*, it appears that the parasite activates its apoptotic machinery when exposed to monoclonal antibody 1D5 and metronidazole. Why does an human intestinal parasite like *B. hominis* undergo PCD/apoptosis? If *B. hominis* were to undergo necrosis *in vivo*, in which dying cells spill their contents into the extracellular space, growth of the whole protozoan population would be negatively affected. We believe that *B. hominis* undergoes apoptosis for its own survival as a population of cells. Some cells undergo apoptosis while the others re-emerge to infect the host when the drug concentration significantly decreases. Some of the insect stages of parasitic trypanosomatids are reported to undergo apoptosis (Moreira *et al.*, 1996; Welburn *et al.*, 1996); despite these parasites existing as clonal populations. It is possible that apoptosis is an ancient method of keeping clonal cell populations in check such that some members are sacrificed to improve the likelihood of successful transmission of the clone as heavy infections could put the survival of the vector at risk (Welburn *et al.*, 1997).

The results of PCD research in *B. hominis* lead one to speculate that cell death mechanisms in this parasite and higher eukaryotes may be conserved (Nasirudeen *et al.*, 2001a, 2001b, 2004a, 2004b). Further detailed research is required in identifying molecules involved in apoptotic mechanisms and regulation of this cell death pathway.

The widespread involvement of apoptosis in physiology and pathology suggests the need for more research in this field. Knowledge of the molecular mechanisms of apoptosis has provided important insights into the causes of diseases where aberrant cell death regulation occurs. Many protozoan infections induce or inhibit apoptosis in mammalian systems. Developing *B. hominis* as a model system for the study of apoptosis would help in the understanding of parasitic apoptotic mechanisms. Moreover, research has shown that the apoptotic mechanism in *B. hominis* closely resembles that of mammalian systems. Further work is required to better delineate the biochemical and molecular control of the apoptotic response and to identify other inducers that cause or inhibit apoptosis contributing to parasitic infections.

Another important thing to note when working with protozoan cell death is the way or manner in which the research is done. Some authors report PCD in parasites with just one or two cell death assays. For example, Picot *et al.*, (1997) reported apoptosis in chloroquine sensitive *Plasmodium falciparum* (the etiological agent of malaria) based on only the occurrence of internucleosomal DNA breakdown. But internucleosomal DNA breakdown or *in situ* DNA fragmentation could also be a result of necrosis (Kok *et al.*, 2002). Apoptosis or PCD cannot be detected with one or two assays but a combination of assays and techniques should be used. Apoptosis or PCD was first

characterized based on morphological characteristics (Kerr *et al.*, 1972). Hence apoptotic/PCD morphological characteristics must first be demonstrated via light/phase contrast microscopy or electron microscopy techniques. Biochemical assays with appropriate controls and early and late time points should then be carried out. Early time points are required to ensure cells are not undergoing necrosis. Time-lapse studies showed that changes in certain cells undergoing apoptosis occurred within 1 to 3h post-induction (Wyllie, 1992) whereas necrosis occurred in a matter of seconds (Collins *et al.*, 1997). Hence, a systematic approach to apoptosis research is necessary to decipher the various cell death pathways involved.

Although protozoan PCD/apoptosis shows morphological and biochemical similarities to cell death in higher vertebrates, molecular signatures differ with no apoptotic gene or protein homologues identified to date. This difference makes PCD research in parasitic protozoa necessary. Understanding the unique molecular aspects of protozoan PCD might lead to drug targets specific for the parasite without causing harm to the mammalian cells. Unlike in mammalian systems, protozoan cell death pathways are poorly understood and hence more research is required to comprehend the complexity in PCD.

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