Apoptosis in *Blastocystis* spp. is related to subtype
D.B. Dhurga, K.G. Suresh *, T.C. Tan, S. Chandramathi

**Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia**

**ARTICLE INFO**

Article history:
Received 25 October 2011
Received in revised form 7 August 2012
Accepted 7 August 2012
Available online 8 November 2012

Keywords:
*Blastocystis* spp.
Apoptosis
Subtypes
Programmed cell death
Metronidazole

**ABSTRACT**

Previous studies have shown that apoptosis-like features are observed in *Blastocystis* spp., an intestinal protozoan parasite, when exposed to the cytotoxic drug metronidazole (MTZ). This study reports that among the four subtypes of *Blastocystis* spp. investigated for rate of apoptosis when treated with MTZ, subtype 3 showed the highest significant increase after 72 h of in vitro culture when treated with MTZ at 0.1 mg/ml (79%; p<0.01) and 0.0001 mg/ml (89%; p<0.001). The close correlation between viable cells and apoptotic cells for both dosages implies that the pathogenic potential of these isolates has been enhanced when treated with MTZ. This suggests that there is a mechanism in *Blastocystis* spp. that actually regulates the apoptotic process to produce higher number of viable cells when treated. Apoptosis may not just be programmed cell death but instead a mechanism to increase the number of viable cells to ensure survival during stressed conditions. The findings of the present study have an important contribution to influence chemotherapeutic approaches when developing drugs against the emerging *Blastocystis* spp. infections.

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1. **Introduction**

Programmed cell death (PCD) is a molecular event that plays a crucial role in the development of multicellular organisms via regulation of their development and growth. PCD involves the physiological non-inflammatory elimination of damaged or harmful cells during organogenesis or for the proper function of continuous cell renewal systems in mature organisms. PCD may take place in any situation among living cells that communicate with each other or with the environment, or both, in an organised manner.

Apoptosis, a common form of PCD, is well characterised by a unique pattern of morphological alterations in the cytoplasm and nucleus. In contrast to necrosis, which normally results in cell rupture releasing the cytotoxic intracellular contents and thus injuring adjacent cells and tissues, apoptosis avoids this damage by compartmentalising the cells into intact, smaller, membrane-bound structures. A number of morphological characteristics of PCD in multicellular organisms can be seen in the apoptosis-like PCD in unicellular protists, including the protozoan parasites *Trypanosoma*, *Leishmania* and *Plasmodium*.

PCD or apoptosis has been investigated in only two intestinal protozoans, namely *Blastocystis* spp. and *Giardia lamblia*. *Giardia* is characterised by abdominal discomfort, acute or chronic diarrhoea, weight loss and dehydration. *Giardia lamblia*, the aetiological agent of giardiasis, undergoes apoptotic-like cell death featuring formation of apoptotic bodies, nuclear fragmentation, chromatin condensation and cytoplasmic vacuolation. Different cell types may not necessarily display all the hallmarks of apoptosis, yet the gold-standard features appear to be conserved in cells undergoing apoptosis, including shrinkage of the cell, preservation of membrane integrity with increasing permeability, nuclear fragmentation and externalisation of plasma membrane phosphatidylserine (PS) residues. Several of these features have been described in *Blastocystis* spp. exposed to cytotoxic drugs...
such as metronidazole (MTZ) and a surface-reactive cytotoxic monoclonal antibody (1DS). Some Blastocystis spp. cells underwent apoptosis; the rest emerged to re-infect the host when the concentration of the drug decreased significantly.7

None of these studies evaluated whether subtype variation could influence the apoptosis rate. Genotypic and phenotypic characterisation of Blastocystis spp. isolates has implicated subtype 3 as having greater pathogenic potential.2 All symptomatic isolates consisting of subtype 3 exhibited the presence of the amoeboid Blastocystis spp. form, a greater size range and multiplied more slowly in Jones’ medium.2 Tan et al.2 confirmed the existence of the amoeboid form, which had been shown to exist in isolates from symptomatic patients in their previous study. This subtype was also associated in patients with irritable bowel syndrome, inflammatory bowel disease and chronic diarrhoea.13

The distinct differences in the phenotypic characterisation previously shown prompted us to investigate whether apoptosis is influenced by subtype, as this information is still lacking. Therefore, the aim of this study was to compare the influence of apoptosis in Blastocystis spp. symptomatic isolate subtype 3 with asymptomatic isolates subtype 1, subtype 2 and subtype 5.

2. Materials and methods

2.1. Parasite culture and subtyping

A total of nine human-derived Blastocystis spp. isolates comprising three symptomatic subtype 3 isolates and six asymptomatic isolates (two each of subtype 1, subtype 2 and subtype 5) were obtained from separate individuals. Blastocystis spp. subtype 3 were isolated from symptomatic patients with diarrhoea and bloating stomach. The other isolates were obtained from infected persons who did not show any symptoms. Isolated parasites were maintained through in vitro cultivation in Jones’ medium supplemented with 10% horse serum and incubated at 37 °C.14 A pea-sized amount of stool was taken from every stool cup and was introduced into culture tubes containing 3 ml of Jones’ medium. Parasites were maintained and were sub-cultured once every 3–4 days for at least 1 month prior to this study. DNA was extracted directly from the culture samples using the QIAGEN Stool Mini Kit (QIAGEN, Hilden, Germany). Then, PCR reaction was performed using the seven pairs of sequenced-tagged site (STS) primers (SB83, SB155, SB227, SB332, SB340, SB336 and SB337). Only isolate of a single subtype is included in this study.16

2.2. Induction of cell death by metronidazole

MTZ (2-methyl-5-nitroimidazole-1-ethanol) is a 5-nitroimidazole drug used for the treatment of anaerobic infections. Stock solutions of MTZ (Discovery Fine Chemicals, Dorset, UK) were prepared in distilled water and were further diluted to obtain the desired concentrations. Then, 1 × 10^5 Blastocystis spp. cells/ml were introduced into a 1.5 ml microcentrifuge tube (Oxygen Biosciences, Union City, CA, USA) containing a final concentration of 0.0001 mg/ml and 0.1 mg/ml MTZ. A microcentrifuge tube containing the same amount of parasites but untreated served as a control. Cells were then harvested at 12, 24, 36, 48, 60, 72, 84 and 96 h for epifluorescence microscopy analysis.

2.3. Cell viability assay

Each time the cells were harvested, cell viability was determined quantitatively by the trypan blue dye exclusion method using a Neubauer haemocytometer chamber (Hauser Scientific, Horsham, PA, USA). Briefly, 10 μl of the cells was mixed thoroughly with 10 μl of dye and allowed to stand for 5 min at 15–30 °C. Cells that were stained and unstained were enumerated as non-viable and viable, respectively.17

2.4. Detection of apoptotic, late apoptotic stage and necrotic cells

Detection of apoptotic, late apoptotic stage and necrotic cells was done using an Apoptosis, Necrotic & Healthy Cells Qualification Kit (Biotium Inc., Hayward, CA, USA). Harvested cells were washed twice with 1 ml of PBS (pH 7.4). Then, 1 × binding buffer, ethidium homodimer III (EtD-III) (200 μM in PBS), fluorescein isothiocyanate (FITC)–annexin V (250 μl in Tris–EDTA buffer containing 0.1% bovine serum albumin and 0.1% NaN3 (pH 7.5)) and Hoechst 33342 (500 μg/ml in PBS) were added sequentially. Samples were then observed under an Olympus BX 51 epifluorescence microscope (Olympus, Wetzlar, Germany) using image analyser software. Results of cells undergoing apoptosis, late apoptosis stage and necrosis were quantified with regard to percentage of apoptotic, late apoptotic stage and necrotic cells per 100 cells. Hoechst 33342, which is a cell-membrane-permeant, minor-groove-binding DNA stain, was used as a substitute for the nucleic acid stain DAPI (4′,6-diamidino-2-phenylindole). It stains the nuclei of both apoptotic and necrotic cells. Apoptotic cells were determined by FITC–annexin V staining, which binds to exposed PS in a cell undergoing apoptosis. Apoptosis causes asymmetrical distribution of the phospholipids located in the inner membrane, and PS is translocated to the outer layer of the plasma membrane.18 PS acts as the recognition signal for annexin V (a PS-binding protein), which thus binds to the exposed PS. This dye is used together with EtD-III, a superior alternative to propidium iodide (PI). Absence of PI staining signals that the membrane integrity is not compromised.19 Healthy cells are stained blue by Hoechst 33342 stain only (Figure 1B). Apoptotic cells are stained with both blue (Hoechst 33342) and green (FITC–annexin V) (Figure 1C). Cells stained blue, green and red (EtD-III) are late apoptotic stage cells (Figure 1D). Cells stained blue and red only are necrotic cells.

2.5. Statistical analysis

Statistical analysis was carried out using SPSS Statistics 18.0 software (SPSS Inc., Chicago, IL, USA). Independent Student’s t-test was used to assess the relationship of
cells undergoing apoptosis between different subtypes. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Cell shrinkage of treated cells

Cell shrinkage is a crucial characteristic feature of apoptosis. In this study, cell shrinkage was determined by measuring the diameter both of the untreated and treated Blastocystis spp. cells. Treated Blastocystis spp. showed a significant decrease in diameter compared with untreated Blastocystis spp. (p<0.001) (Table 1), thus demonstrating that cell shrinkage does take place in parasites from each subtype exposed to the drug.

3.2. Viable and apoptotic cells

Subtype 3 showed a significantly higher increase in the rate of apoptosis at 72 h in comparison with other asymptomatic subtypes (subtypes 1, 2 and 5) (Figure 2D,F), where 89% (p<0.001) and 79% (p<0.01) of cells showed apoptosis for drug concentrations of 0.0001 mg/ml and 0.1 mg/ml, respectively (Table 2). In fact, both the high and low doses showed a significant increase (p<0.001) in the apoptosis rate and cell viability. There was a clear correlation between viable and apoptotic cells for subtype 3 (Table 3). Cells treated with 0.0001 mg/ml and 0.1 mg/ml MTZ showed a higher correlation value (r=0.948 and r=0.915, respectively) compared with untreated cells (r=0.688).

Table 1

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Untreated cells</th>
<th>Treated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0001 mg/ml</td>
<td>0.1 mg/ml</td>
</tr>
<tr>
<td>1</td>
<td>25 ± 9.836</td>
<td>12 ± 1.166&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>26 ± 12.235</td>
<td>14 ± 5.133&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>24 ± 11.475</td>
<td>13 ± 4.236&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>23 ± 11.435</td>
<td>14 ± 4.167&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD.
<sup>a</sup> p<0.001, comparing the diameter of treated cells with untreated cells.

4. Discussion

Loss of cell or cytoplasmic volume is a fundamental characteristic of cells undergoing apoptosis. When cell shrinkage or a reduction in cell volume occurs, the cell becomes smaller, the cytoplasm becomes denser and the organelles become more tightly packed, thus decreasing the diameter of the cell. MTZ causes apoptotic-like cell death in Blastocystis spp., where light microscopy data showed cell shrinkage or a reduction in size of the cell. In this study, a reduction in the diameter of drug-treated cells supports the evidence that induction of apoptosis via MTZ has taken place. Measurement of the diameter of treated cells from each subtype showed a significant reduction compared with untreated cells.

In this study, the high number of apoptotic cells that picked up the FITC-annexin V dye and were seen as green cells under epifluorescence microscopy shows that the drug used in this study to induce apoptosis would have triggered more PS to be expressed on the surface of the cell. Both a high and low concentration of the drug showed a higher elevation level of apoptosis rate at 72 h (Table 2). The significant elevation (Table 2) in the rate of apoptosis of isolates belonging to subtype 3 in the treated condition showed that symptomatic isolates are more prone to apoptosis in comparison with asymptomatic isolates. This study has also reported a positive correlation (Table 3) between apoptosis and cell viability, since increased cells undergoing apoptosis show a corresponding increase in viable cells. In a report by Haresh et al., it was concluded that drug-treated parasites show a larger number of granular forms. These forms could have been implicated in the possible release of the reproductive granules and thus increases in the number of viable parasites in culture. It is highly probable that pathogenicity could be influenced by cell numbers. Perhaps that is why, as the apoptosis rate increases, cells that are becoming viable in subtype 3 are significantly greater in comparison with isolates from other subtypes.

The occurrence of cell suicide responses has already been reported in unicellular eukaryotic cells and prokaryotic cells. Apoptosis may not just be PCD but instead a mechanism that triggers an increase in the numbers of viable cells to ensure survival during stressed conditions. In unicellular organisms, PCD selects the fittest cell in the
population and further regulates the process of cell cycle and cell differentiation of the entire population. A good example would be the sporulation process of Bacillus subtilis. During conditions that are not conducive to survival, B. subtilis divides symmetrically to make two daughter cells (binary fission), or asymmetrically to make a daughter cell and a mother cell. While the mother cell initiates a process of differentiation, ultimately leading to cell death, the daughter cell will be a long-lived spore capable of repopulating a bacterial colony. Asexual reproduction by means of binary fission is known in diatoms, which belong to the complex group of protozoa called stramenopiles. Stramenopiles includes unicellular and multicellular protists, and other members are brown algae, chrysophytes, water moulds, slime nets and the recently classified Blastocystis. This further postulates that there is a mechanism involved in Blastocystis spp. that actually regulates the apoptotic process to produce a higher number of viable cells of the parasite in response to treatment. Further detailed research at the molecular level may need to be carried out to elucidate apoptosis mechanisms and the cell death pathway in Blastocystis spp.

Apoptosis in unicellular organisms could be a defence mechanism for the whole population. The type of stimuli used to induce cell death need to be studied. Environmental stimuli can produce various types of cell death depending on the intensity of the stimulus.

To date, MTZ is still used as the drug of choice in the treatment of infections caused by protozoans. Several studies have reported on the resistance of Blastocystis spp. isolates to MTZ. In another study by Jones et al., nine of 21 symptomatic patients were positive for

Figure 2. (A,C,E) Number of viable cells and (B,D,F) rate of apoptosis in Blastocystis spp.: (A,B) untreated cells; (C,D) cells treated with 0.0001 mg/ml metronidazole; and (E,F) cells treated with 0.1 mg/ml metronidazole. Subtype 3 showed a significantly higher increase in the rate of apoptosis at 72 h compared with the other asymptomatic subtypes (subtypes 1, 2 and 5), where 89% (p<0.001) and 79% (p<0.01) of cells showed apoptosis at drug concentrations of 0.0001 mg/ml and 0.1 mg/ml, respectively. In fact, both the high and low doses showed significant elevation (p<0.001) in the apoptosis rate and cell viability. st: subtype.
Table 3

Correlation between viable and apoptotic Blastocystis spp. subtype 3 cells in untreated and treated (0.0001 mg/ml and 0.1 mg/ml metronidazole) conditions after 72 h of culture

<table>
<thead>
<tr>
<th>Treatment</th>
<th>r-value*</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0.688</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Treated at 0.0001 mg/ml</td>
<td>0.948</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treated at 0.1 mg/ml</td>
<td>0.915</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Cells treated with metronidazole at 0.0001 mg/ml and 0.1 mg/ml showed a higher correlation value compared with untreated cells.

Blastocystis spp., of whom six were infected with Blastocystis spp. subtype 3. In that study, most of the patients reported failure with MTZ. It is highly probable that the findings in the present in-vitro study could provide the basis for observations in in-vivo studies where treatment with MTZ has failed. The findings in the present study are an important contribution to influences on chemotherapeutic approaches to the development of drugs against the emerging Blastocystis spp. infections.

Authors’ contributions: DBD, KGS and TCT were involved in intellectual planning of the study; DBD and KGS designed the study; DBD carried out the experiments; TCT and SC contributed to the molecular aspects of the study; DBD, KGS and SC analysed the data and prepared the manuscript; KGS, TCT and SC edited the paper. All authors read and approved the final manuscript. KS is guarantor of the paper.

Acknowledgements: The authors thank the staff at the Department of Parasitology, Faculty of Medicine, University of Malaya (Kuala Lumpur, Malaysia).

Funding: Funding for this study was provided by a High Impact Research Grant (UMC/625/1/HIR) and by the University of Malaya (grant PS237/2010 B).

Competing interests: None declared.

Ethical approval: This study was approved by the Medical Ethics Committee of the University of Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia, according to the Declaration of Helsinki.

References


