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Research paper

Microvesicles released from *Giardia intestinalis* disturb host-pathogen response *in vitro*

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ABSTRACT

Giardia intestinalis (G.I), is an anaerobic protozoan and the aetiological agent of giardiasis, a diarrhoea present worldwide and associated with poverty. G.I has a simple life cycle alternating between cyst and trophozoite. Cysts are transmitted orally to the stomach and transform to trophozoites in the intestine by a multifactorial process. Recently, microvesicles (MVs) have been found to be released from a wide range of eukaryotic cells. We have observed a release of MVs during the life cycle of G.I., identifying MVs from active trophozoites and from trophozoites differentiating to the cyst form. The aim of the current work was to investigate the role of MVs from G.I in the pathogenesis of giardiasis. MVs from log phase were able to increase the attachment of *G. intestinalis* trophozoites to Caco-2 cells. Moreover, MVs from *G. intestinalis* could be captured by human immature dendritic cells, resulting in increased activation and allostimulation of human dendritic cells. Lipid rafts participate in the MV biogenesis and in the attachment to Caco-2 cells. Nevertheless, proteomic analysis from two types of MVs has shown slight differences at the protein levels. An understanding of biogenesis and content of MVs derived from trophozoites might have important implications in the pathogenesis of the disease.

1. Introduction

Cell-cell communication is mediated by secreted biomolecules, including peptides, proteins, lipids and nucleic acids. These molecules are also present in extracellular vesicles (EVs: mainly exosomes and microvesicles), which are released from different cell types and are able to bind to receptors on target cells, triggering intracellular signalling that modifies the physiological state of the target cells (Ratajczak et al., 2006).

EVs are found at elevated levels in cancer and in different acute and chronic inflammatory diseases including sepsis, stroke, atherosclerosis and diabetes mellitus (reviewed in Lower et al., 2014; Aurelian et al., 2014). They are also found in physiological processes such as coagulation (Julich et al., 2014).

Recently, many authors have described the involvement of EVs during the parasite-host interaction. These authors have shown the presence of EVs of different sizes carrying microRNAs, proteins and pro-inflamatory cytokines modulating the host cell (Marcilla et al., 2014; Evans-Osses et al., 2015; Barteneva et al., 2013)

As the leading cause for protozoal diarrhoea worldwide, the intestinal parasite *Giardia intestinalis* (Syn *G. duodenalis*, *G. lamblia*) is an important pathogen of humans and animals causing morbidity and adversely affecting economies. *Giardia* has a peculiar biology and represents an interesting biological model to under-

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stand evolution, organelle function, and antigenic variation (Adam, 2001).

Giardia intestinalis has two evolutionary stages, the trophozoite, which is located in the gut of animals and humans and which multiplies by binary fission, and the infectious stage, the cyst, released into the environment in faeces.

Giardia belongs to the phylum Diplomonadida, unicellular eukaryotes that have undergone considerable reductive evolution. The lateral gene transfer (LGT) mechanism, an important evolutionary step in prokaryotes, has been shown in *Giardia*, supporting this parasite to be included in an early branch of eukaryotic evolution (Embley and Hirt, 1998). These findings provide insights into the evolution of biochemical pathways in early eukaryote evolution, and could be important in understanding the minimization, or even loss, of most cellular systems such as mitochondria, peroxisomes, Golgi apparatus, and a classical endo-lysosomal system.

Pathological cysts are ingested via the oral route and symptoms usually occur after an incubation period of 1–2 weeks, although half of *Giardia* infections are asymptomatic. After emergence from cysts, the flagellated *G. lamblia* trophozoites colonize mainly the upper small intestine. Trophozoites reside and replicate in the intestinal lumen and at intestinal epithelial cells, but are not able to invade the mucosa. Although still poorly understood, it is clear that the attachment of the parasite to the mucosal surface is the critical point for its persistence in the host. The parasite contains a ventral disk that seems to be important for attachment (Woessner and Dawson, 2012), while the flagella contributes to correct positioning and orientation of the trophozoites before the attachment (House et al., 2011). The parasite actively engages mucosal immunity and the infection progresses with a low or absent inflammation in most cases (Oberhuber and Stolte, 1997). Most likely, mechanical effects, or some other, as yet not described mechanism, produces villus and brush border microvillus atrophy, leading to digestive enzyme deficiencies (Solaymani-Mohammadi and Singer, 2011), and chronic giardiasis can lead to mucosal inflammation with pronounced villus loss (Hanevik et al., 2007); protease activities may be a direct cause of diarrhoeas in giardiasis. Moreover, Jiménez et al. (2004) found that excretory and secretory antigens (E/S Ags) from *G. lamblia* induced an intestinal pathogenesis, which coincided with mucosal inflammation in BALB/c mice. Oral administration of the E/S Ags not only stimulated production of antibodies with parasiticidal activity, but also resulted in histological alterations within the intestinal tissue that were comparable to those observed in natural and experimental *Giardia* infections (Jiménez et al., 2014). After colonization the cyst formation represents a key step in the life cycle of the parasite. This process involves cellular and molecular events. Luján et al. (1996) reported that cholesterol starvation induces encystation.

Three encystation- specific cyst wall proteins (CWP1, 2 and 3) are expressed and concentrated in encystation-specific- vesicles (ESVs) that circulate within the parasite before being transported to the cyst wall (Luján et al., 1996; Reiner et al., 1990; Lauwaet et al., 2007; Faso and Hehl, 2011; Benchimol and De Souza, 2011). Synthesis of ESVs starts 4–6 h after encystation is induced and is completed with the cyst formation by approximately 24 h (Reiner et al., 1990). Interestingly, the protozoan could be able to release other kinds of vesicles that could be speculated to be associated with the attachment to the intestinal cells and pathogenesis. In preliminary work (Deolindo et al., 2013) we have shown that *G. Intestinalis* may release MVs when exposed to different pHs and inducers. Now, We have continued an in depth analysis of MV biogenesis and of the phenotype of the extracellular vesicles released by the parasite.

We have hypothesized in this work that the response of *Giardia intestinalis* to environmental stress conditions results in the active release of MVs from the plasma membrane that modulate the host-parasite cell interaction.

2. Materials and methods

2.1. Cell culture

A human colonic adenocarcinoma cell line, Caco-2 cell clone C2BBe1 [30], was obtained from the American Type Culture Collection (CRL-2102). Caco-2 cells (passages 57–72) were cultured at 37 °C, 5% CO_2 in Dulbecco's Modified Eagle's medium (DMEM; Cellgro, Manassas, VA) supplemented with 10% foetal bovine serum (FBS) (Life Technologies, Grand Island, NY), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were fed every third day and passaged using 0.025% trypsin with 0.22 mM EDTA when 80–90% confluent.

2.2. Parasite culture and in vitro encystation

Giardia lamblia strain WB clone C6 was obtained from the American Type Culture Collection (#50803). Parasites were grown in filter sterilized modified TYI-S-33 medium with 10% adult bovine serum and 0.05% bovine bile at 37 °C in microaerophilic conditions and subcultured when confluent. To collect parasites for experiments, the medium was removed from the culture to eliminate unattached or dead parasites. The tube was refilled with cold, sterile medium and trophozoites detached by chilling on ice for 15 min. Parasites were collected by centrifugation (1500 × *g* for 5 min at 4 °C) and washed once with the plating medium of 90% complete DMEM/10% Giardia medium. Parasites were then counted using a hemocytometer, and diluted to the appropriate number.

Encystation was induced as described previously (McCaffery and Gillin, 1994). Briefly, the pre-encysting cultures were grown to late log phase for 48 h in TYI-S-33 medium (pH 7.1) without antibiotics. Encystation was initiated by removing the spent medium and non-adherent cells and re-nourishing the adherent cells with an encystation medium (TYI-S-33 medium adjusted to pH 7.8 and supplemented with 0.25 mg/ml bovine bile and 5 mM lactic acid).

2.2.1. Inhibition of lipid rafts

Trophozoites from *G. intestinalis* stationary phase were decanted, washed and ressuspended in TYI-S-33 medium without FBS. The parasites were incubated with 2.5, 5.0 and 10 μM de MβCD for 1 h at 37C. After this time the parasites were centrifugated at 1000 × *g* for 10 min and the pellet ressuspended with fresh TYI-S-33 medium and used in microvesiculation and adhesion essays.

2.2.2. Adhesion assay

The assay was carried out with stationary phase cultures of *G. Intestinalis* trophozoites or with *G. intestinalis* trophozoites treated with MβCD (10 μm). The parasites were decanted by chilling for 10 min in ice-cold PBS, at pH 7.2. Trophozoite suspensions were centrifuged at 1000 × *g* for 10 min and resuspended to a concentration of 1×10^6/ml. Caco cells were seeded on a coverslip in a concentration of 1×10^5 cells/well. Suspensions of tropho zoites were then co-incubated with cultured cells in a 10:1 ratio. Plates were incubated at 37 °C in 10% CO_2. After incubating for 1–3 h, unattached trophozoites were counted in a haemocytometer and the% adhered cells determined. The effect of vesicles was tested in the same experiments incubated with different concentrations of purified MVs.

2.3. Monocyte isolation

Cells were obtained from leukocyte residues of healthy donors from the Blood and Tissue Bank (Barcelona, Spain). Peripheral blood