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Research Brief

Trypanosoma cruzi: Participation of cholesterol and placental alkaline phosphatase in the host cell invasion

S. Priotto [a], M.J. Sartori [a,b], G. Repossi [a,c], M.A. Valentich [a,b,c,*]

[a] Instituto de Biología Celular, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, CP5000, CC 220, Ciudad Universitaria, Córdoba, Argentina
[b] Cátedra de Biología Celular, Histología y Embriología, Instituto de Biología Celular, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, CP5000, CC 220, Ciudad Universitaria, Córdoba, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

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ABSTRACT

One of the most important parasitic endemic diseases in Latin America is Chagas disease, with almost 20 million people being infected and 60 million others at risk of infection. In the cell infection by *Trypanosoma cruzi*, the first step is contact with the host cell by receptors and ligands on the membrane. It is known that lipids play an important role in the interaction process between pathogens and host cells with lipid rafts being highly specialized regions of the plasma membrane that are enriched in cholesterol and sphingolipids. We explored whether the treatment with methyl-beta-cyclodextrin alone or by adding Mevinolin, an inhibitor of cholesterol synthesis could deplete membrane cholesterol of the HEp2 cell and if this treatment could affect the trypomastigote infection into the host cell. These treatments led to a leakage of cholesterol, and concomitantly, PLAP enzyme and unidentified proteins resulting in a decrease of the invasion process. However, the GGTP enzyme was not liberated from the host cell membranes.

1. Introduction

In order to invade target cells, metacyclic forms of *Trypanosoma cruzi* and blood trypomastigotes engage a plethora of surface and secreted molecules, some of which are involved in triggering the specific signaling pathways both in the parasite and the host cell, critical for parasite entry (Alves and Colli, 2007). Recently, it was suggested that membrane rafts, including caveolae, are involved in the process of *T. cruzi* invasion of macrophages (Barrias et al., 2007). Related to this, a role may be played by placental alkaline phosphatase (PLAP)[1] (EC: 3.1.3.1), a plasma membrane glycoenzyme, present in the human placental trophoblast and also in HEp-2 cells. This enzyme is modified in the serum of pregnant women infected as well as in human placental villi cultured with *T. cruzi* (Sartori et al., 1997). It is anchored to the membrane by a gly-cosylphosphatidylinositol (GPI) molecule, and inserted in lipidic microdomains (Giocondi et al., 2007) and can be solubilized by phospholipase C (PL-C), a molecule secreted by the parasite (Fernandez et al., 2006). Once released, the GPI-anchored enzymes might activate transmembrane signaling (Sharom and Radeva, 2004).

Another enzyme, gamma glutamyltranspeptidase (GGTP) (EC: EC 2.3.2.2), is also membrane located and involved in stress processes (Paolicchi et al., 2002) but it is has not yet been studied with respect to *T. cruzi* invasion. Although, it has been reported that the kinetic parameters of this enzyme change with the dietary lipids, nothing is known about its association with membrane lipids (Valentich and Monis, 1990; Medina Basso et al., 2006).

We decided to examine if cholesterol, PLAP and GGTP enzymes of Hep-2 cells, and parasitic invasion could be affected after the use of a novel strategy in *T. cruzi* cell invasion (as Fernandes et al. (2007) applied in Hela o Vero cells) by depleting cholesterol from specialized membrane microdomains, designated membrane rafts, as a possible portal to introduce the parasite into host cells.

2. Materials and methods

2.1. Materials

Penicillin, streptomycin, MβCD, Menivolin and minimum essential medium (MEM) were provided by Sigma Chemical Co., St. Louis, USA; foetal bovine serum (FBS) and the reactives for the determination of cholesterol and the enzyme activities by local suppliers.

* Corresponding author. Address: Cátedra de Biología Celular, Histología y Embriología, Instituto de Biología Celular, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, CP5000, CC 220, Ciudad Universitaria, Córdoba, Argentina.
E-mail address: mirtavalentich@gmail.com (M.A. Valentich).

[1] *Abbreviations used:* PLAP, placental alkaline phosphatase; GGTP, gamma glutamyltranspeptidase; MβCD, methyl beta cyclodextrin; Mev, Mevinolin; GPI, glycosylphosphatidylinositol.

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2.2. Cell culture

The HEp2 cells used in this work were from the American Type Cell Collection (Hep-2 cells: http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452). Cells were grown for at least for 24 h using minimum essential medium (MEM) supplemented with 10% FBS, penicillin (100 Ul/mg), streptomycin (100 μg/ml), in a 5% CO_2 humid atmosphere to obtain a semiconfluent monolayer before each assay.

2.3. Isolation of trypomastigotes

Trypomastigotes from the Tulahuen strain of *T. cruzi* were isolated from blood Albino Swiss mice at the peak of parasitemia, according to Andrews and Colli (1982). Pellets containing the parasites were washed twice and resuspended in minimum essential medium (MEM).

2.4. Cholesterol depletion

For cholesterol depletion, cells were washed twice with MEM culture medium and incubated at 37 °C for 0, 30 and 60 min with 10 mM MβCD (Sigma Chemical Co., St. Louis, USA) in MEM and with 10 mM of HEPES at pH 7.5 (without serum and Fenol Red), as described by Hunter and Nixon (2006). After treatment, cells were collected with scrapers and centrifuged at 800*g* for 1 min. The pellets of cells obtained were used to determine the PLAP activity through zymmogram detection plus densitometry, and the supernatant was used for colorimetric cholesterol, protein and enzyme activity determinations. Cell viability after MβCD treatment was confirmed by proliferating activity in complete medium with cells being able to proliferate as efficiently as before the cholesterol depletion.

2.5. Cholesterol determination

The cholesterol was quantified in culture medium MEM with 10 mM of HEPES at pH 7.5 (without serum and Fenol Red) of treated and control cells using an oxidase/peroxidase enzymatic commercial kit (Gamble et al., 1978).

2.6. Determination of alkaline phosphatase activity

The alkaline phosphatase activity was analyzed in the pellets of treated cells by a zymmogram after centrifugation at 100*g* for 5 min at 4 °C, and the specific activity was determined in sonicated culture medium without BFS according to a technique used by Sartori et al. (2003). This was expressed as μmol of *p*-nitrophenol per mg of protein per min, with the protein level being measured using the method of Bradford (1976).

2.7. Determination of gamma glutamyltranspeptidase

The gamma glutamyltranspeptidase activity was estimated in cells and culture medium with the modified Szasz method, following the Gamma-G-test kinetic AA kit manufacturer's instructions (Szasz, 1969). Samples were mixed with 100 mM Tris–HCl substrate buffer pH 8.5, containing 2.9 mM L-gamma-glutamyl-3-carboxi-4-nitroanilide and 100mM GLY–GLY in the proportion 1:9 (v/v). The absorbance at 405 nm was recorded and results were expressed in μmol of the product (*p*-nitroaniline liberated) per mg of protein per min at pH 8.5 and 37 °C.

2.8. Zymmogram to detect PLAP activity

HEp2 cell proteins were separated in SDS–PAGE according to Laemmli (1970) with 3% stacking gel and 10% resolving gel. After electrophoresis, the gel was incubated with 50 mg of alpha-naphthyl acid phosphatase monosodium salt, 25 mg of Fast Blue RR salt and Cl_2 Mg 50 mM in 60 ml of buffer Tris–HCl 1.2 M, at pH 9 and 37 °C for 15 min according to the method described by Mezzano et al. (2005).

2.9. Invasion assay

Parasite invasion was determined by detecting it inside the host cells, as currently used in our laboratory with brief modifications (Sartori et al., 2003). Infection assays were carried out in cultures reaching a density of 5×10^3 cells by well. Semiconfluent cells were washed three times with PBS and a solution containing the desired number of parasites (20 parasites/cell) diluted in MEM was added to the groups: (1) control (without any pre-treatment), (2) pre-treated with MβCD and (3) pre-treated with the MβCD plus 2 μM Mevinolin. Parasites and cells were left to interact for 45 min at 37 °C in a 5% CO_2 humid atmosphere, after which the cultures were thoroughly washed with PBS to remove free parasites before been fixed with cold methanol for 15 min and colored with Toluidine Blue to detect the presence of the parasite inside the cells.

2.10. Statistical analysis

The data obtained were analyzed by ANOVA tests and Fisher post-hoc test. A value of less than 0.05 ($P < 0.05$) was chosen to define significant differences.

3. Results and discussion

It is known that the PLAP enzyme is anchored to the membrane by a GPI molecule, with it also has been implicated in the transmembrane and also intracellular signals (Shin and Abraham, 2001). Previous results of our laboratory were consistent with a pathogenetic role of PLAP in congenital Chagas disease (Sartori et al., 2003, 2005; Lin et al., 2002, 2005; Mezzano et al., 2005). On the other hand, a previous report from our laboratory showed that GGTP, another membrane enzyme, changed with the membrane lipid composition. However, nothing is known if GGTP is associated with some specific membrane lipid, with microdomains enriched in cholesterol (Valentich and Monis, 1990; Medina Basso et al., 2006) or with *T. cruzi* infection.

Recent studies have shown that lipids, especially domains known as lipid rafts, play an important role in the interaction process between different pathogens and their host cells (Duncan et al., 2004; Zaas et al., 2005). In order to investigate any the possible involvement of cholesterol enriched lipid microdomains, we performed experiments that consisted of treating HEp2 cells with MβCD or MβCD + Mevinolin, and then evaluating the effect on the cholesterol, PLAP and GGTP depletion, and parasite invasion.

Cholesterol depletion induced liberation of proteins and PLAP but not the GGTP enzyme. MβCD treatment caused, concomitantly, the depletion of both cholesterol and PLAP enzyme from the HEp2 cell membrane, which confirms the association of the enzyme in cholesterol enriched microdomains. Cells treated with MβCD released significant cholesterol into culture medium at 30 min with the rate of liberation slowing down. Furthermore, proteins and PLAP enzyme were also released (Fig. 1). The pattern of liberation of the PLAP enzyme from the Hep2 cells was similar to that of cholesterol, increasing significantly at the time of MβCD treatment with liberation rate being higher at 0–30 than 30–60 min (Fig. 1). However, under the same treatment, the enzyme GGTP was not depleted from the membrane cells to the culture media (Fig. 1). The activity of the enzyme PLAP of cells treated with MβCD was detected by optical density of