Available online at www.sciencedirect.com
ScienceDirect
International Journal for Parasitology 37 (2007) 1431–1441
www.elsevier.com/locate/ijpara

Novel strategy in *Trypanosoma cruzi* cell invasion: Implication of cholesterol and host cell microdomains

Maria Cecília Fernandes [a], Mauro Cortez [a], Kelly Aparecida Geraldo Yoneyama [b], Anita Hilda Straus [b], Nobuko Yoshida [a], Renato Arruda Mortara [a,*]

[a] *Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo - Escola Paulista de Medicina, Rua Botucatu, 862, 6º andar, São Paulo, SP 04023-062, Brazil*

[b] *Departamento de Bioquímica, Universidade Federal de São Paulo - Escola Paulista de Medicina, Rua Botucatu, 862 São Paulo, SP 04023-062, Brazil*

Received 2 April 2007; accepted 24 April 2007

Abstract

Trypanosoma cruzi, the etiological agent of Chagas' disease, is an obligatory intracellular parasite in the mammalian host. In order to invade a wide variety of mammalian cells, *T. cruzi* engages parasite components that are differentially expressed among strains and infective forms. Because the identification of putative protein receptors has been particularly challenging, we investigated whether cholesterol and membrane rafts, sterol- and sphingolipid-enriched membrane domains, could be general host surface components involved in invasion of metacyclic trypomastigotes and extracellular amastigotes of two parasite strains with distinct infectivities. HeLa or Vero cells treated with methyl-β-cyclodextrin (MβCD) are less susceptible to invasion by both infective forms, and the effect was dose-dependent for trypomastigote but not amastigote invasion. Moreover, treatment of parasites with MβCD only inhibited trypomastigote invasion. Filipin labeling confirmed that host cell cholesterol concentrated at the invasion sites. Binding of a cholera toxin B subunit (CTX-B) to ganglioside GM1, a marker of membrane rafts, inhibited parasite infection. Cell labeling with CTX-B conjugated to fluorescein isothiocyanate revealed that not only cholesterol but also GM1 is implicated in parasite entry. These findings thus indicate that microdomains present in mammalian cell membranes, that are enriched in cholesterol and GM1, are involved in invasion by *T. cruzi* infective forms.

Keywords: *Trypanosoma cruzi*; Trypomastigote; Amastigote; Membrane rafts; Cholesterol; Cell invasion

1. Introduction

The involvement of plasma membrane microdomains in the entry process and intracellular replication of microbial pathogens is becoming increasingly evident. Cellular invasion by viruses (Pelkmans, 2005) and the uptake of a great number of intracellular bacteria into professional or non-professional phagocytic cells (Gulbins et al., 2004; Lafont and van der Goot, 2005) has been shown to require a specialized host membrane microenvironment, organized into membrane rafts. These microdomains are small (10–100 nm), heterogeneous, highly dynamic, sterol- and (glyco)sphingolipid-enriched domains that compartmentalize cellular processes (Simons and Ehehalt, 2002; Pike, 2006). Membrane rafts were originally proposed to be involved in the sorting of proteins to the apical surface of polarized epithelial cells, with which specific proteins interact (Simons and Ikonen, 1997). Cholesterol is thought to serve as a spacer between the hydrocarbon chains of sphingolipids and to function as a dynamic adhesive that keeps the raft assembly together (Simons and Toomre, 2000). The role of cholesterol and membrane rafts in the uptake of larger pathogens like protozoa is beginning to be addressed. Host cell cholesterol is recruited during *Toxoplasma gondii* invasion (Coppens and Joiner, 2003) and the closely related apicomplexan parasite *Plasmodium falciparum* targets sphingomyelin and cholesterol to the parasitophorous vacuole (Lauer et al., 2000). Recently, it has also been shown

* Corresponding author. Tel.: +55 11 5579 8306; fax: +55 11 5571 1095.
E-mail address: ramortara@unifesp.br (R.A. Mortara).

0020-7519/$30.00
doi:10.1016/j.ijpara.2007.04.025

that *Leishmania donovani* uptake into macrophages also depends on host cell cholesterol, since its depletion with nystatin or methyl-β-cyclodextrin (MβCD) inhibits the process (Pucadyil et al., 2004; Tewary et al., 2006).

The implication of host cell cholesterol or membrane microdomains in the entry process of *Trypanosoma cruzi* has not yet been addressed. *Trypanosoma cruzi* is the etiological agent of Chagas' disease and it is known that the parasite is able to infect a wide variety of mammalian host cells (Brener, 1973; De Souza, 2002). However, this ability to enter non-phagocytic mammalian cells may vary widely between strains and infective forms (Ruiz et al., 1998; Neira et al., 2002; Fernandes and Mortara, 2004; Silva et al., 2006; Yoshida, 2006). Numerous studies have been performed in order to understand the molecular mechanisms that underlie the rather complex process of parasite entry into mammalian host cells. Unfortunately, some of these have proposed mechanisms that turned out not to be as general as initially supposed (Ortega-Barria and Pereira, 1991; Tardieux et al., 1992; Ming et al., 1995) and until now attempts to completely abolish host cell colonization have not succeeded.

The variety of invasion strategies engaged by *T. cruzi* metacyclic trypomastigotes as well as extracellular amastigotes is consistent with the complex repertories of surface molecules that both infective forms of the parasite have evolved to ensure infection (reviewed in Mortara et al., 2005; Yoshida, 2006). The search for specific protein host cell receptors involved in *T. cruzi* invasion has turned out to be a laborious and challenging task (Yoshida, 2006), so we decided to examine the possibility that lipids, abundant and widespread in host cell membranes, could play a role in this process. In the present work we have evaluated the ability of the parasite to invade cholesterol-depleted cells as well as the ability of sterol-depleted parasites to invade untreated cells. We demonstrate, for the first time, the involvement of cholesterol in the *T. cruzi* entry process. Host cell cholesterol was shown to be recruited to the invasion site of both infective forms studied and pre-treatment of HeLa and Vero cells with cholesterol sequestering agent, MβCD, inhibited parasite invasion. In addition, binding of a cholera toxin B subunit (CTX-B) to GM1, a marker of membrane rafts, significantly decreased parasite infectivity, indicating that not only cholesterol but also membrane rafts participate in this process.

2. Materials and methods

2.1. Cells and parasites

Vero and HeLa cell lines obtained from Instituto Adolpho Lutz (São Paulo, SP, Brazil) were cultivated in Dulbecco's modified Eagles medium (DMEM) (Cultilab, Campinas, SP, Brazil) with 10% FCS (Cultilab) in a humid atmosphere of 5% CO_2 at 37 °C. Cell-derived trypomastigotes from G (Yoshida, 1983) and CL (Brener and Chiari, 1963) strains were obtained after infection of semi-confluent Vero cell lines. Cells grown in 75 cm^2 flasks were infected with recently released cell-derived trypomastigotes (10^8 parasites/ml). Infection proceeded overnight at 37 °C in DMEM supplemented with 10% FCS. The supernatant was then replaced by DMEM 2% FCS and cells were kept at 37 °C. Tissue culture-derived trypomastigotes emerge from Vero cells after approximately 6 days of infection. Extracellular amastigotes were obtained from the differentiation of cell-derived trypomastigotes isolated in liver infusion tryptose (LIT) – define medium. Vero cell-derived trypomastigotes were isolated from culture supernatants of infected cells by centrifugation at 2500*g* for 5 min. The pellet was resuspended in LIT medium, pH 5.8, and incubated for 24–48 h at 37 °C, and at least 95% pure extracellular amastigotes were obtained (Ley et al., 1988; Mortara, 1991). Metacyclic trypomastigotes from both strains were obtained from the axenic differentiation of stationary-phase hemocultures of mice previously infected with tissue-culture trypomastigotes, in liver infusion tryptose (LIT, Camargo, 1964) containing 5% FCS and 0.2% glucose, or Grace's medium (CL strain) at 28 °C and purified by ionic exchange chromatography (Yoshida, 1983).

2.2. Cell invasion assays

Semiconfluent Vero or HeLa cells were infected with metacyclic trypomastigotes or amastigotes at a proportion of 10:1 parasites/cell (CL strain trypomastigotes and G strain amastigotes) and 25:1 parasites/cell (G strain trypomastigotes and CL strain amastigotes). After 1 h, DMEM supplemented with 5% FCS was removed and cells were washed five times with PBS. Cells were then fixed with either 3.5% formaldehyde in PBS for filipin labeling, or with methanol for Giemsa staining. The number of intracellular parasites were counted in 500 cells in duplicate coverslips. The percentage of infectivity was calculated according to the formula: number of internalized parasites × 100/number of intracellular parasites in control cells.

2.3. Cholesterol depletion and repletion

For cholesterol depletion, cells were washed twice with PBS and incubated at 37 °C for 45 min with MβCD (Sigma Chemical Co., St. Louis, USA) at the indicated concentration in DMEM (without serum). Cell viability after MβCD treatment was confirmed by 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2*H*-tetrazolium bromide (MTT) reaction (Mosmann, 1983). For cholesterol repletion, Vero cells were incubated with the indicated concentration of water soluble cholesterol (Sigma Chemical Co., St. Louis, USA) in DMEM without serum for 30 min, or 90 min incubation with DMEM with 10% or 20% FCS. After the procedure, cells were washed twice with PBS before adding the parasites. Infection was performed as described above.