Available online at www.sciencedirect.com
ScienceDirect
Micron 39 (2008) 25–31
micron
www.elsevier.com/locate/micron

Immunolocalization of an osteopontin-like protein in dense granules of *Toxoplasma gondii* tachyzoites and its association with the parasitophorous vacuole

Erika Cortez [a], Ana Carolina Stumbo [a], Roberta Saldanha-Gama [b], Christina Gaspar Villela [b], Christina Barja-Fidalgo [b], Carlos Alberto Rodrigues [c], Maria das Graças Henriques [c], Marlene Benchimol [d], Helene S. Barbosa [e], Luis Cristóvão Porto [a], Laís Carvalho [a,*]

[a] *Laboratório Cultura de Células, Departamento de Histologia e Embriologia, IB, Universidade do Estado do Rio de Janeiro, UERJ, Av. Prof. Manoel de Abreu 444, 3° andar, 20550-170, Rio de Janeiro, RJ, Brazil*
[b] *Departamento de Farmacologia, IB, UERJ, RJ, Brazil*
[c] *Laboratório de Farmacologia Aplicada, Fiocruz, RJ, Brazil*
[d] *Laboratório de Ultra-estrutura Celular, USU, RJ, Brazil*
[e] *Laboratório de Biologia Estrutural, IOC, Fiocruz, RJ, Brazil*

Received 16 July 2007; received in revised form 20 August 2007; accepted 30 August 2007

Abstract

Toxoplasma gondii is an apicomplexan parasite infecting a broad host range, including humans. The parasite invades host cell by active penetration with the participation of its secretory organelles proteins during this process. Until now, only a limited number of secretory proteins have been discovered, and the effectors molecules involved in parasite invasion and survival are not well understood. Osteopontin (OPN) is a multifunctional glycophosphoprotein, secreted by different cell types, which is involved in various physiological and pathological events including cell signaling and survival. For the first time we demonstrated in this work by immunofluorescence and immunoelectron microscopy approaches the localization of an OPN-like protein in dense granules of extracellular *T. gondii* tachyzoites. Western blotting and RT-PCR confirmed this protein expression by the parasites. Our results also showed, after macrophage invasion, an intense positive labeling for OPN-like protein at the sub-apical portion of tachyzoites, the site of dense granules secretion, and the localization of this protein at the parasitophorous vacuole membrane. These data suggest that dense granules secrete an OPN-like protein, and we speculate that this protein participates during the parasite interaction process with host cells and parasitophorous vacuole formation.

Keywords: *Toxoplasma gondii*; Tachyzoites; Dense granules; Osteopontin; Host cell; Macrophage

1. Introduction

Toxoplasma gondii is an obligate intracellular pathogen infecting a variety of host cells where it multiplies within a specially modified compartment called parasitophorous vacuole (PV) (de Souza, 2005; Lemgruber et al., 2007; Martin et al., 2007). Inside PV, *T. gondii* stays free from many of the host's immune insults that are encountered by extracellular microbes. This feature crucially relies upon successful entry into a target cell and avoidance of host cell defenses response such as acidification or endolysosomal hydrolases (Cortez et al., 2005; Carruthers and Boothroyd, 2007).

T. gondii tachyzoites invade host cell by active penetration, a carefully coordinated process driven by the parasite actin and myosin machinery and independent of host cell (Black and Boothroyd, 2000; Kim, 2004). The process begins with gliding motility that utilizes a reversible attachment to migrate over the surface of the host cell, perhaps to find a susceptible site for entry (Heintzelman, 2006). The parasite then establishes an intimate association, involving reorientation to put the apical secretory structures in contact with the host membrane. Next, the specialized secretory organelles: micronemes, rhoptries and dense granules, in the parasite apical end are secreted in a precisely orchestrated series of events during invasion

* Corresponding author. Tel.: +55 21 25876125.
E-mail address: ldc29@globo.com (L. Carvalho).

0968-4328/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.micron.2007.08.007

(Cesbron-Delauw et al., 1996; Carruthers and Sibley, 1997; Brossier and Sibley, 2005; Henriquez et al., 2005; Dubremetz, 2007).

Dense granules (DG) are microspheres of approximately 200 nm in diameter, surrounded by a unique membrane, found in the invasive stages of Apicomplexa. Secretion of DG occurs at a sub-apical location of the parasite by fusion of the DG membrane with the parasite plasma membrane (Mercier et al., 2005). The existence of two mechanisms driving secretion from DG is hypothesized. The first mechanism is a constitutive release (Liendo et al., 2001) and the second mechanism, occurring during the first hour following host cell invasion and regulated by an uncharacterized mechanism (Mercier et al., 2005). The DG proteins (GRA) are a group of relatively small proteins (21–41 kDa). An interesting common feature of GRA proteins is the difference observed between their theoretical molecular weight, calculated from the amino acid sequence and the molecular weight of the native protein, estimated by SDS-PAGE analysis of tachyzoite lysates. This suggests potential post-translational modifications such as phosphorylation and glycosylation (Zinecker et al., 1998; Mercier et al., 2005).

Osteopontin (OPN) is a multifunctional protein that was first identified as a major sialoprotein in bone (Prince and Butler, 1987) and subsequently found to be expressed by several cell types, including macrophages, fibroblasts and myofibroblasts (Murry et al., 1994; Hartner et al., 2001; Li et al., 2002; Pereira et al., 2006). OPN is a secreted adhesive glycophosphoprotein that contains 314 amino acids with a predicted molecular mass of 32 kDa (Oldberg et al., 1986; Denhardt et al., 2001). However, like GRA proteins, there are substantial post-translational modifications, including phosphorylation and glycosylation resulting in a protein with electrophoretic mobility between 44 and 75 kDa (O'Regan, 2003). OPN is involved in various physiological and pathological events including cell survival, cell signaling, cell-mediated immunity, cellular adhesion, cellular differentiation, inflammation, tissue repair and tumor metastasis (Standal et al., 2004; Pereira et al., 2006; Rangaswami et al., 2006).

Only a limited number of *T. gondii* secretory proteins have been identified (Zhou et al., 2005). Moreover, the effectors molecules involved in parasite invasion and survival are not well understood. Our aim in this study was evaluate the expression profile of OPN in *T. gondii* tachyzoites and its participation during the interaction parasite–host cell.

2. Materials and methods

2.1. Parasites

Tachyzoites from the virulent RH strain of *T. gondii* were maintained by intraperitoneal passages in female Swiss mice. The parasites were collected 72 h after infection in phosphate buffer saline (PBS), pH 7.2. The suspension obtained from infected mice was centrifuged at 200 × *g* for 10 min at room temperature to completely remove cells and debris, and the supernatant, which only contained the parasites, was collected and centrifuged at 1000 × *g* for 10 min. The pellet obtained was washed 2 or 3 times with PBS, pH 7.2 and resuspended in Dulbecco's modified Eagle's medium (DMEM). The parasites were used within 30–40 min after removal from the mouse peritoneal cavity, and the viability was evaluated using dye-exclusion test with Trypan blue (Carvalho and de Souza, 1989). The procedures with animals were carried out in accordance with the guidelines established by the Fundação Oswaldo Cruz FIOCRUZ, Committee of Ethics for the Use of Animals, by license CEUA 0229-04.

2.2. Macrophage cultures

Resident macrophages were collected from peritoneal cavities of Swiss mice after injection of PBS, pH 7.2 and then plated on glass coverslips in 24-well plates. After incubation for 1 h at 37 °C, the non-adherent cells were removed and DMEM containing 10% fetal calf serum was added. The monolayers of macrophages were cultivated for 24 h at 37 °C in a 5% CO_2 atmosphere and then, used for the experiments.

2.3. Osteopontin-like protein expression analysis by confocal laser scanning microscopy

T. gondii tachyzoites on coverslips pre-coated with poly-L-lysine (Sigma Chemical Co., St. Louis, Missouri) or macrophage cultures infected with *T. gondii* for 15 min, 30 min, 1 h and 24 h were fixed with 4% paraformaldehyde in PBS, pH 7.2. Then, the cells were permeabilized for 15 min in PBS containing 0.5% Triton X-100 and indirectly immunolabeled with a monoclonal anti-osteopontin rat primary antibody (1:100; Chemicon International, Inc., Temecula, CA, USA) followed by a FITC conjugated anti-rat secondary antibody (Sigma). Controls were performed by omission of the monoclonal primary antibody. The cells were observed under an Olympus BX 51 microscope coupled with an Olympus CLSM FV 300 Fluoview Version 3.3.

2.4. Osteopontin-like protein detection by immunoelectron microscopy

Extracellular *T. gondii* tachyzoites were collected and fixed with 0.2% glutaraldehyde, 2% paraformaldehyde, and 0.1% picric acid in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h at room temperature. Afterwards cells were washed in PBS, dehydrated in ethanol and embedded in LRWhite resin (Sigma). For immunolabeling, ultrathin sections were washed with PBS/BSA, quenched with 50 mM ammonium chloride for 30 min, and subsequently incubated overnight with the monoclonal anti-osteopontin rat primary antibody (1:10, Chemicon). The sections were washed with PBS/BSA followed by 1 h incubation with a 10 nm gold-conjugated anti-rat secondary antibody (1:20, Sigma), washed, stained with uranyl acetate, and observed under an EM 906 Zeiss transmission electron microscope. Controls were performed by omission of the monoclonal primary antibody.