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Original article

Interaction of epithelial cell membrane rafts with *Paracoccidioides brasiliensis* leads to fungal adhesion and Src-family kinase activation

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Abstract

Membrane rafts are cholesterol- and sphingolipid-enriched cell membrane domains, which are ubiquitous in mammals and play an essential role in different cellular functions, including host cell-pathogen interaction. In this work, by using several approaches, we demonstrated the involvement of epithelial cell membrane rafts in adhesion process of the pathogenic fungus *Paracoccidioides brasiliensis*. This conclusion was supported by the localization of ganglioside GM1, a membrane raft marker, at *P. brasiliensis*-epithelial cell contact sites, and by the inhibition of this fungus adhesion to host cells pre-treated with cholesterol-extractor (methyl-β-cyclodextrin, MβCD) or -binding (nystatin) agents. In addition, at a very early stage of *P. brasiliensis*-A549 cell interaction, this fungus promoted activation of Src-family kinases (SFKs) and extracellular signal-regulated kinase 1/2 (ERK1/2) of these epithelial cells. Whereas SFKs were partially responsible for activation of ERK1/2, membrane raft disruption with MβCD in A549 cells led to total inhibition of SFK activation. Taking together, these data indicate for the first time that epithelial cell membrane rafts are essential for *P. brasiliensis* adhesion and activation of cell signaling molecules.

Keywords: Membrane microdomains; *Paracoccidioides brasiliensis*; Host-pathogen interactions; Adhesions; Src-Family Kinases; ERK MAP kinases

1. Introduction

Paracoccidioidomycosis is a human deep mycosis, which occurs mostly in South and Central America [1]. This disease is characterized by a granulomatous inflammation with clinical forms ranging from a benign localized infection to a disseminated one, affecting several parts of the body. Nowadays, it is accepted that infection with *Paracoccidioides brasiliensis*, the etiological agent of paracoccidioidomycosis, occurs by inhaling mycelial forms of this fungus, with subsequent conversion of mycelial to yeast forms and establishment of infection in host lungs [1].

Over the past 15 years, the notion that pathogens hijack host cell signaling for their own survival has become an undeniable concept. For mycopathogens, some studies reported host cell signaling pathway modulation by fungal infection. Belanger et al. [2] and Monteiro da Silva et al. [3], for example, showed that invasion of *Candida albicans* and *P. brasiliensis*, respectively, was associated with activation of host

Abbreviations: Brij®98, polyoxyethylene(20) oleyl ether; BSA, bovine serum albumin; CTB, cholera toxin subunit B; DAPI, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl-sulfoxide; DRM, detergent-resistant membrane; ERK1/2, extracellular signal-regulated kinase 1/2; HRP, horseradish peroxidase; IMix, mixture of inhibitors containing Na_3VO_4, leupeptin, aprotinin and Pefabloc®; M-PER®, Mammalian-Protein Extraction Reagent; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; MβCD, methyl-β-cyclodextrin; PBS, phosphate buffered saline; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; Pefabloc®, 4-(2-aminoethyl)benzenesulfonyl-fluoride-hydrochloride; P-ERK1/2, phospho-ERK1/2; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine; P-SFK, phospho-SFK; PVDF, polyvinylidene-difluoride; SFK, Src-family kinase; TBST, 200 mM Tris buffer pH 8.0 containing 150 mM NaCl and 0.05% Tween-20; TNE, 25 mM Tris buffer pH 7.5 containing 150 mM NaCl and 5 mM EDTA pH 7.5.

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cell tyrosine kinases. Moreover, other host cell signaling molecules, involved in different cell events, such as apoptosis, may be activated or blocked depending on the fungus studied (see review ref. [4]).

Despite these data obtained through fungal-host cell interactions, more advanced researches have been accomplished with bacteria, virus and protozoa showing that these microorganisms are able to adhere, invade and proliferate by manipulating different host cell signaling pathways, which may involve activation of certain kinases [5]. For example, internalization of bacteria like *Listeria monocytogenes*, one of the etiological agent of gastroenteritis, requires activation of Src kinase and other host cell signaling molecules, such as cortactin and Arp2/3 [6]. *Plasmodium falciparum*, one of the causative species of malaria, also promotes Src-family kinase activation which seems essential for the adhesion of infected erythrocytes to host endothelium [7]. Although the functional role remains to be elucidated for *P. falciparum*-host interaction, the same authors observed that the recombinant peptide PpMC-179 (corresponding to the minimal endothelial CD36-binding domain from *P. falciparum* erythrocyte membrane protein1 – PfEMP1), in a Src-dependent manner, activated extracellular signal-regulated kinase 1/2 (ERK1/2) [7]. On the other hand, different studies suggest that activation of ERK1/2 is involved in internalization of several bacteria such as *Pseudomonas aeruginosa* [8], *L. monocytogenes* [9] and *Campylobacter jejuni* [10]. Moreover, membrane rafts of host cell, recently defined as small (10–200 nm), highly dynamic and heterogeneous membrane domains which are enriched in cholesterol and sphingolipids [11], seem to be essential for the infection events of some pathogens, such as *P. aeruginosa*, *Shigella flexneri* and *P. falciparum*, leading to modulation of host cell signaling events, and, consequently, to their internalization and intracellular survival (see review ref. [12]).

Since adhesion is a crucial step for pathogens to establish an infection in mammalian hosts, in the present work, we investigated whether yeast forms of *P. brasiliensis* promote activation of Src and ERK1/2 kinases during early interaction of this fungus with human lung epithelial cells. We also analyzed the importance of host membrane rafts in *P. brasiliensis* adhesion to epithelial cells.

2. Materials and methods

2.1. Fungal growth conditions

P. brasiliensis, strain 18, was kindly provided by Dr. Zoilo P. Camargo, São Paulo, Brazil. Yeast forms were grown 5–7 days at 37 °C, 100 rpm, in PGY (Peptone/Glucose/Yeast extract) medium as described before [13].

2.2. Cell culture

Human lung epithelial cell line A549 and African green monkey kidney epithelial cell line Vero were grown in DMEM (Sigma, MO, USA), supplemented with 10% fetal bovine serum (Cultilab, SP, Brazil), 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin (complete DMEM) at 37 °C, 5% CO_2.

2.3. Effect of epithelial cell cholesterol sequestration on P. brasiliensis association

A total of 3×10^4 A549 or Vero cells were cultured on 12 mm diameter glass coverslips for 2 days. For cholesterol sequestration, cells were washed twice with serum-free DMEM, and then, incubated with this medium containing 10 mM methyl-β-cyclodextrin (MβCD – Sigma, MO, USA) or 25 μg/ml nystatin (Sigma, MO, USA) at 37 °C, for 1 h or 30 min, respectively. For some experiments, cholesterol was replenished by removing MβCD and adding 1 mM cholesterol-MβCD complex (water-soluble cholesterol – Sigma, MO, USA) for 30 min. Next, cells were washed with serum-free DMEM and incubated with 4.5×10^5 yeast forms of *P. brasiliensis*. After 3 h, cells were sequentially: i) washed with warm phosphate buffered saline (PBS, 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl) supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS^+); ii) fixed with 4% paraformaldehyde for 10 min; iii) washed with PBS^+; and iv) stained with Wright-Giemsa-like Hema 3 kit following manufacturer's instructions (Fisher, MI, USA). Controls were performed: i) in the absence of MβCD or cholesterol; or ii) in the presence of 0.5% DMSO as solvent control for nystatin. All experiments were performed in triplicate, analyzing 300 cells per coverslip. Statistical significance was evaluated by Student's *t*-test.

A549 or Vero cell viability was measured with Trypan Blue 0.2% in PBS or MTT assay as described before [14]. Briefly, after treatment with MβCD, nystatin or cholesterol, A549 or Vero cells were washed with DMEM without phenol red or HEPES. Next, cells were incubated for 3 h with the same medium containing 0.5 mg/ml MTT. After medium removal, MTT formazan was solubilized with 200 μl of DMSO. Cell viability was determined at 540 nm with a Labsystems Multiskan MCC/340 microplate reader. DMSO solution was used as blank reference.

2.4. GM1 cell localization by fluorescence microscopy

Cells were cultured, and then, incubated with *P. brasiliensis* as described above. After 5 h, cells were washed three times with PBS^+ to remove unbound yeasts, fixed with 4% paraformaldehyde for 10 min, and incubated sequentially with 5% bovine albumin (BSA) in PBS, and 125 ng/ml cholera toxin subunit B (CTB) AlexaFluor®488-conjugate (Invitrogen, OR, USA) in PBS containing 5 μM DAPI (Sigma, MO, USA), 1 μg/ml calcofluor (Sigma, MO, USA) in 1% BSA. After each step, cells were washed three times with PBS^+. Coverslips were mounted onto glass slides with SlowFade® (Invitrogen, OR, USA) reagent and fluorescence was analyzed using an epifluorescence microscope.