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Original article

Paracoccidioides brasiliensis induces secretion of IL-6 and IL-8 by lung epithelial cells. Modulation of host cytokine levels by fungal proteases

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Abstract

Paracoccidioides brasiliensis is a pathogenic, dimorphic fungus that causes paracoccidioidomycosis, a systemic human mycosis that is highly prevalent in Latin America. In this study, we demonstrated that *P. brasiliensis* yeasts induced interleukin (IL)-8 and IL-6 secretion by human lung epithelial A549 cells. However, tumor necrosis factor-α and interferon-γ were undetectable in these cultures. Moreover, *P. brasiliensis* yeasts induced activation of p38 mitogen-activated protein kinase (MAPK), c-Jun NH_2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) 1/2 in A549 cells, and IL-8 and IL-6 secretion promoted by this fungus was dependent on activation of p38 MAPK and ERK 1/2. In addition, IL-8 and IL-6 levels were significantly higher in culture supernatants of A549 cells that were incubated with formaldehyde-fixed *P. brasiliensis* compared to cultures of cells that were infected with live yeasts. Our results indicate that the observed cytokine level differences were due to protease expression, in live yeasts, that degraded these cytokines. Degradation of human recombinant IL-8 and IL-6 by live *P. brasiliensis* was inhibited by AEBSF and aprotinin, suggesting that these proteases belong to a family of serine proteases. This is the first report showing that *P. brasiliensis* may modulate host inflammation by expressing proteases that degrade proinflammatory cytokines.

Keywords: *Paracoccidioides brasiliensis*; Cytokines; Epithelial cells; p38 MAPK; MAPK-ERK kinases; Proteases

1. Introduction

The dimorphic fungus *Paracoccidioides brasiliensis* is the etiological agent of paracoccidioidomycosis (PCM), which is the most prevalent systemic fungal infection in Brazil and is also present in some other Latin America countries (Venezuela, Colombia, Ecuador and Argentina) [1–3]. An estimated 10 million people are infected with this fungus, and 1–2% of infected individuals will develop PCM [1,3]. Clinically, this mycosis is often characterized by a slow and progressive disease, predominantly affecting lungs and mucocutaneous tissue. In some cases, the disease disseminates throughout the patient's body, infecting central nervous system, adrenal glands, lymph nodes, liver, spleen and other organs [3,4].

Pulmonary epithelial cells constitute one of the first lines of defense against inhaled particles or microorganisms. These cells are able to secrete cytokines and chemokines, indicating that epithelial cells also play an important role in host innate immunity [5,6]. Several studies were performed with bacteria showing that this response is somewhat species-specific, because different cytokine secretion profiles are observed depending on the pathogen studied. For example, Chang et al. [7] showed that *Legionella pneumophila* induces secretion of interleukin (IL)-8, IL-6 and tumor necrosis factor-alpha (TNF-

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α) by the human lung epithelial cell line A549. *Mycoplasma pneumoniae* also promotes IL-8 and TNF-α secretion, but IL-6 and interferon-γ (IFN-γ) levels are not increased during *M. pneumoniae*-A549 cell interaction [8]. However, the interaction of *Mycobacterium tuberculosis* with these epithelial cells causes an increase in IFN-γ levels [9].

Cytokine secretion was also evaluated during fungus-epithelial cell interaction, and these reports are mostly focused on *Candida albicans* and *Aspergillus fumigatus*. Several groups have demonstrated that *C. albicans* induces secretion of IL-1α/β, IL-6, granulocyte-colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), IL-8, TNF-α and RANTES by oral epithelial cells (see review Ref. [10]). Regarding *A. fumigatus*, different groups have demonstrated that proteases or germinating conidia of *A. fumigatus* induce the secretion of IL-8 by bronchial or lung epithelial cell lines [11,12]. In addition, Balloy et al. [11] verified that IL-8 synthesis in human bronchial epithelial cells (BEAS-2B), during *A. fumigatus* infection, is dependent on the activation of phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinases (MAPKs) such as p38 MAPK and extracellular signal-regulated kinase 1/2 (ERK 1/2).

Pathogens may promote activation of one or more of the three subfamilies of MAPKs (p38 MAPK, ERK and c-Jun NH_2-terminal kinase − JNK) in epithelial cells, and this cell signaling is frequently related to secretion of cytokines and chemokines. Recently, Schmeck et al. [13] demonstrated that *L. pneumophila* induces the secretion of at least 10 cytokines by A549 cells. These authors verified that secretion of most of these cytokines (IL-4, IL-6, IL-8, IL-17, monocyte chemoattractant protein-1 − MCP-1, TNF-α, IL-1β and IFN-γ) is dependent on p38 MAPK activation, while secretion of a few of them (IFN-γ, IL-1β, IL-6 and TNF-α) is dependent on ERK 1/2 activation. Regarding IL-8 secretion, Carmona et al. [14] demonstrated similar results with *Pneumocystis carinii* cell wall β-glucans (PCBG). They showed that PCBG promotes IL-8 secretion by human airway epithelial cells ($1HAEo^-$), which is dependent on activation of p38 MAPK and ERK 1/2 but independent of JNK activation. On the other hand, IL-8 secretion by human intestinal epithelial cells (INT-407) infected with enteroaggregative *Escherichia coli* is dependent on JNK activation, in addition to p38 MAPK and ERK 1/2 activation [15]. Taken together, these results indicate that secretion of a specific cytokine (e.g. IL-8) by epithelial cells depends on different MAPK pathways that are activated according to epithelial cell type and pathogen species, reflecting, in this manner, the interaction between distinct host receptors and pathogen molecules.

In addition to exploiting host cell signaling, some pathogens, such as *Porphyromonas gingivalis*, may also manipulate host immune system by secreting proteases, which can degrade cytokines, chemokines, immunoglobulins, complement components or cytokine receptors (see review Ref. [16]). Only *Candida* sp. and *A. fumigatus* have been described as fungal microorganisms that express proteases that degrade proteins of host immune system, specifically of the complement system [17].

Recently, we observed that *P. brasiliensis* yeasts promote activation of ERK 1/2 during early interaction of this fungus with human lung epithelial A549 cells [18]. In the present study, we first analyzed whether *P. brasiliensis* induced secretion of proinflammatory cytokines by A549 cells. We also verified the involvement of different MAPKs in modulating *P. brasiliensis*-induced cytokine release by these epithelial cells. Finally, we showed that *P. brasiliensis* yeasts express proteases that are able to degrade cytokines.

2. Materials and methods

2.1. Fungal growth conditions

P. brasiliensis, strain Pb18, was kindly provided by Dr. Zoilo P. Camargo, São Paulo, Brazil. Yeasts were grown as described previously [19]. Briefly, fungal aliquots were cultivated in PGY (neopeptone 5 g/l, glucose 15 g/l, yeast extract 5 g/l, asparagine 1.4 g/l and thiamine 0.1 g/l) for 5–7 days in an incubator shaker at 37 °C, 100 rpm.

2.2. A549 cell culture

Human lung epithelial cell line A549 was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Vitrocell Embriolife, Brazil), 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin (complete DMEM) at 37 °C, 5% CO_2.

2.3. Preparation of P. brasiliensis yeasts for interaction assays with A549 cells or incubation with recombinant IL-6 or IL-8

First, *P. brasiliensis* yeasts, grown for 5–7 days, were decanted for 30 min. The resultant supernatant contained only single mother and small daughter yeasts. Then, yeasts were washed four times with PBS (10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl). Depending on the assay, yeasts were then fixed, heat-killed or kept alive. To prepare live yeasts of *P. brasiliensis*, yeasts were washed two more times with DMEM. For formaldehyde-fixed yeasts, *P. brasiliensis* was fixed with PBS containing 4% formaldehyde for 10 min, washed twice with PBS, and then washed twice with DMEM. For heat-killed yeasts, yeasts were incubated at 80 °C for 2 h, and then washed twice with DMEM. Next, yeasts were resuspended in DMEM and incubated with A549 cells or human recombinant (hr) IL-6 or IL-8 as described in items 2.4–2.6. Efficiency of killing *P. brasiliensis* yeasts by formaldehyde or heat was confirmed by the inability of these fungi to grow in PGY.

2.4. Analysis of MAPKs activation during the interaction of A549 cells with P. brasiliensis

Approximately 2.0×10^6 A549 cells were cultured in 100 mm plates with complete DMEM. After 1 day, cells were incubated overnight in FBS-free DMEM to decrease basal