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Ultrastructural characterization of melanosomes of the human pathogenic fungus *Fonsecaea pedrosoi*

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Abstract

Melanin is a complex polymer widely distributed in nature and has been described as an important virulence factor in pathogenic fungi. In the majority of fungi, the mechanism of melanin formation remains unclear. In *Fonsecaea pedrosoi*, the major etiologic agent of chromoblastomycosis, melanin is stored in intracellular vesicles, named melanosomes. This paper details the ultrastructural aspects of melanin formation, its storage and transportation to the cell wall in the human pathogenic fungus *F. pedrosoi*. In this fungus, melanin synthesis within melanosomes also begins with a fibrillar matrix formation, displaying morphological and structural features similar to melanosomes from amphibian and mammalian cells. Silver precipitation based on Fontana-Masson technique for melanin detection and immunocytochemistry showed that melanosome fuses with fungal cell membrane where the melanin is released and reaches the cell wall. Melanin deposition in the fungal cell wall occurs in concentric layers. Antibodies raised against *F. pedrosoi* melanin revealed the sites of melanin production and storage in the melanosomes. In addition, a preliminary description of the elemental composition of this organelle by X-ray microanalysis and elemental mapping revealed the presence of calcium, phosphorus and iron concentrated in its matrix, suggesting a new functional role for these organelles as iron storage compartments.

Keywords: *Fonsecaea pedrosoi*; Melanin; Cell wall; Melanosome; X-ray microanalysis

1. Introduction

Melanins are phenolic and heterogeneous biopolymers, produced and distributed among a wide range of organisms, which plays a role between protection and virulence, and possibly contributes to the maintenance of several species along evolution (Plonka and Grabacka, 2006). In pathogenic fungi, the synthesis and expression of these pigments have been associated with microbial virulence (Gomez et al., 2001). Melanized fungi have been shown to be more resistant to host defenses. Several features of melanin appear to be involved in fungal resistance, such as scavenging of free radicals, capacity to interact with antifungal drugs preventing them from reaching their target sites, absorption of UV light, protection against enzymatic lysis, desiccation and extreme variations of temperature (Butler and Day, 1998). Due to its complexity, difficulty extraction/isolation, and diversity, the exact structure and composition of any melanin is still unknown.

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In fungi, the study of melanin synthesis, storage and transport could potentially provide important insights for the rational development of active drugs that could positively interfere with this strong microbial virulence factor (Nosanchuk and Casadevall, 2003).

In mammalian cells, the biosynthesis of melanin occurs through a cation-dependent mechanism in unique membrane-enclosed structures of melanocytes and retinal pigmented epithelial cells named melanosomes (Salceda and Riesgo-Escovar, 1990). The mammalian melanogenesis process begins with the formation of premelanosomes, vesicular structures with internal membranes without melanin (stage I). This organelle differentiates into elongated structures with internal striations (stage II). Melanin is then accumulated within the striations, resulting in their thickening and blackening (stage III) until this pigment fills the entire melanosome (stage IV). The melanosomes are then either stored or secreted and incorporated by other cell types, such as the keratinocytes (Seiji et al., 1963a,b; Marks and Seabra, 2001).

The *in vitro* and *in vivo* production of melanin was recently demonstrated in important human pathogenic dimorphic fungi such as *Blastomyces dermatitidis* (Nosanchuk et al., 2004), *Paracoccidioides brasiliensis* (Gomez et al., 2001), *Sporothrix schenckii* (Morris-Jones et al., 2003), *Penicillium marneffei* (Youngchim et al., 2005), *Histoplasma capsulatum* (Nosanchuk et al., 2002) and *Coccidioides posadasii* (Nosanchuk and Casadevall, 2006).

In fungal pathogens, melanin biosynthesis is usually described as occurring at the cell wall and being derived from phenolic compounds or intermediates. However, Alviano et al. (1991) showed that in the black fungi *Fonsecaea pedrosoi*, melanin is synthesized within membrane bounded structures referred to as melanosomes. Similar organelles were described in other black fungi, such as *Cladosporium carrioni* and *Hormoconis resinae* (San-Blas et al., 1996).

Fonsecaea pedrosoi is a dematiaceous and medically important pathogenic fungus in tropical countries and is the most frequent etiologic agent of chromoblastomycosis, an endemic subcutaneous mycosis commonly observed in the Amazon region (Bonifaz et al., 2001; Silva et al., 1999). It produces melanin constitutively and has been considered as a model of melanin-producing fungi (Alviano et al., 2004; Cunha et al., 2005; Franzen et al., 1999, 2006). In contrast to other fungal species that need external precursors (L-Dopa, e.g.) to synthesize this pigment (Chaskes and Tyndall, 1975; Kojeg et al., 2004; Williamson, 1994), *F. pedrosoi* synthesizes melanin constitutively through the polyketide pathway, a process that starts with acetyl-CoA (Jacobson, 2000).

The ability of melanin to bind transition metals and to quench free radicals is thought to be an important factor of virulence (Jacobson, 2000). Iron is essential for oxidation-reduction reactions (Byers and Arceneaux, 1998) and has been previously described as an important constituent of fungal melanin and responsible for their protection against oxidative mechanisms (Henson et al., 1999). In microorganisms, acidic conditions are necessary for such metals to bind to polyphosphate (Docampo et al., 2005). The production of melanin inside melanosomes of *F. pedrosoi* occurs in an acidic environment, as previously revealed by the accumulation of acridine orange inside these organelles (Franzen et al., 1999). The estimated intra-melanosomal pH in mammalian cells is around 3–5, melanin biosynthesis is reduced at neutral pH as major enzymes involved in melanogenesis have no activity at this pH (Bathnagar et al., 1993).

Calcium is a ubiquitous intracellular second messenger that regulates a wide range of cellular activities (Berridge et al., 2000). The accumulation of calcium and melanin in animal melanosomes suggests that melanin granules could perhaps serve as a calcium reservoir (Salceda and Riesgo-Escovar, 1990; Salceda and Sanchez-Chavez, 2000).

In this report, the ultrastructure of melanosomes of *F. pedrosoi* was further analyzed by transmission electron microscopy. Analysis of thin sections showed the four stages of melanogenesis in the melanosomes of *F. pedrosoi*, sharing similar features of amphibian and mammalian melanosomes described by Prelovsek and Bulog (2003) and Raposo and Marks (2002). Preliminary analysis of the elemental content of the melanosomes by electron probe X-ray microanalysis and elemental mapping showed the presence of phosphorus, iron and calcium concentrated in the melanosomal matrix. Interestingly, the same elements have been found in the cell wall. Cytochemical and immunocytochemical detection of melanin showed the concentration of this polymer in the melanosomes and in the cell wall, where it was organized in concentric layers. In addition, transport and release of melanosomal content to the cell wall, through a fusion mechanism of the melanosomes with the plasma membrane, was observed. Altogether, the results contribute for the further understanding of the functional role of this organelle in pathogenic fungi.

2. Materials and methods

2.1. Chemicals

The majority of the reagents and organic solvents were purchased from Merck (Rio de Janeiro, RJ, Brazil). Gold-labeled goat anti-human IgG antibody was purchased from Pelco (Redding, CA, USA).

2.2. Microorganism and growth conditions

A human isolate of *F. pedrosoi* (strain 5VLP, Oliveira et al., 1973) was used in the present work. Stock cultures were maintained on Saubouraud's glucose agar medium under mineral oil and kept at 4 °C. Conidia were obtained from the stock culture and incubated at 28 °C for 5 days in Czapeck-Dox modified medium (CDM, each indicated as $g \times L^{-1}$: sucrose 30, $NaNO_3$ 2, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.5, ammoniacal iron citrate 0.01), under shaking