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Melanin dependent survival of *Apergillus fumigatus* conidia in lung epithelial cells

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ABSTRACT

Aspergillus fumigatus is the most important air-borne pathogenic fungus of humans. Upon inhalation of conidia, the fungus makes close contact with lung epithelial cells, which only possess low phagocytic activity. These cells are in particular interesting to address the question whether there is some form of persistence of conidia of *A. fumigatus* in the human host. Therefore, by also using uracil-auxotrophic mutant strains, we were able to investigate the interaction of A549 lung epithelial cells and *A. fumigatus* conidia in detail for long periods. Interestingly, unlike professional phagocytes, our study showed that the presence of conidial dihydroxynaphthalene (DHN) melanin enhanced the uptake of *A. fumigatus* conidia by epithelial cells when compared with non-pigmented *pksP* mutant conidia. Furthermore, conidia of *A. fumigatus* were able to survive within epithelial cells. This was due to the presence of DHN melanin in the cell wall of conidia, because melanised wild-type conidia showed a higher survival rate inside epithelial cells and led to inhibition of acidification of phagolysosomes. Both effects were not observed for white (non-melanised) conidia of the *pksP* mutant strain. Moreover, in contrast to *pksP* mutant conidia, melanised wild-type conidia were able to inhibit the extrinsic apoptotic pathway in A549 lung epithelial cells even for longer periods. The anti-apoptotic effect was not restricted to conidia, because both conidia-derived melanin ghosts (cell-free DHN melanin) and a different type of melanin, dihydroxyphenylalanine (DOPA) melanin, acted anti-apoptotically. Taken together, these data indicate the possibility of melanin-dependent persistence of conidia in lung epithelial cells.

Introduction

Aspergillus fumigatus is an opportunistic human pathogenic fungus, found ubiquitously in nature. The airborne spores are constantly inhaled and reach the lung alveoli due to their small size. In healthy individuals the conidia are efficiently cleared. However, in humans suffering from an impaired immune system, the conidia can germinate and thereby cause severe infections (for an overview see Brakhage, 2005; Dagenais and Keller, 2009). Lung epithelial cells represent the first cellular line of interaction between the fungus and the host and are supposed to play a role for the progress and development of a fungal infection (Filler and Sheppard, 2006). To date, there is only scant information about the interplay between epithelial cells and conidia, in particular when intracellular processing is concerned.

Apoptosis is a key process in host innate immunity. This programmed cell death is triggered extracellularly or intracellularly by two major signalling pathways, *i.e.*, the extrinsic or death receptor mediated pathway and the intrinsic or mitochondrial mediated pathway. Both pathways involve the activation of caspases (Martinou and Green, 2001), which are responsible for biochemical and morphological changes associated with apoptosis (Taylor et al., 2008). Caspase-3 is a central element of both apoptotic pathways. However, a caspase-independent apoptotic pathway has also been reported (Chose et al., 2003; Susin et al., 2000). Many pathogens are able to modulate host cell apoptosis as a mechanism of defense. Some pathogens such as the yeast *Candida*

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albicans induce host cell apoptosis to evade phagocytic killing and penetrate the epithelial barrier (Ibata-Ombetta et al., 2003) while other pathogens like *Leishmania sp.* inhibit host cell apoptosis to survive in an intracellular niche to facilitate its spreading (Ruhland et al., 2007). Therefore, manipulation of host cell apoptosis is an important strategy of pathogens to establish infection of the host (Rodrigues et al., 2012; Rudel et al., 2010).

Conidial dihydroxynaphthalene (DHN) melanin of *A. fumigatus* was previously identified as an important virulence determinant when tested in murine infection models for aspergillosis (Langfelder et al., 1998; Tsai et al., 1998). DHN melanin was shown to inhibit apoptosis in macrophages. Conidia of the non-melanised *pksP* mutant, which lack the polyketide synthase involved in DHN melanin biosynthesis, are not able to sustain macrophage survival upon treatment with an intrinsic apoptotic inducer (Volling et al., 2011). In addition to its anti-apoptotic property, melanin suppresses the host immune responses of macrophages and neutrophils, *e.g.*, by protecting conidia against reactive oxygen intermediates (ROI) (Brakhage and Liebmann, 2005; Jahn et al., 1997, 2000), reducing complement activation (Behnsen et al., 2008; Tsai et al., 1997) and inhibiting intracellular processing of conidia by reduction of phagolysosomal acidification (Jahn et al., 2002; Thywissen et al., 2011). Recent studies have demonstrated that *A. fumigatus* also inhibits host cell apoptosis in different types of epithelial cells (Berkova et al., 2006). However, to date, the fungal factor responsible for the inhibitory effect on epithelial cell apoptosis remained elusive.

Since epithelial cells only have reduced phagocytic activity, it is reasonable to assume that at least a few conidia might survive within these cells. Such conidia could represent the infectious reservoir as soon as the host's immune system becomes impaired. Here, we show that conidia of *A. fumigatus* are able to survive within epithelial cells. Interestingly, only melanised conidia but not white (non-melanised) conidia of the *pksP* mutant strain inhibited apoptosis in lung epithelial cells. Furthermore, *A. fumigatus* is able to prevent phagosomal acidification in alveolar epithelial cells and to survive intracellularly, suggesting the possibility of persistence.

Materials and methods

Fungal strains and culture conditions

In addition to the *A. fumigatus* wild-type strain CEA10 (CBS144.89) two uracil auxotrophic *A. fumigatus* strains were used: CEA17 (D'Enfert et al., 1996) which is a derivative of CEA10 characterized by a point mutation in the *pyrG* gene and CEA17pksP, which is a non-melanised mutant obtained by partial deletion of the *pksP* gene in CEA17. *A. fumigatus* was cultivated on *Aspergillus* minimal medium (AMM) agar plates with 10 mM uracil and 5 mM uridine as described previously (Weidner et al., 1998). Conidia were harvested in sterile 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20, filtered through 40 μm pore size cell strainers (BD Biosciences) and washed with phosphate buffered saline (PBS) supplemented with 0.1% (v/v) Tween 20 (PBST).

Cell lines and growth conditions

The human type II pneumocyte cell line A549 (ATCC-CCL-185™) was used for all experiments. Cells were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in F-12K medium (LGC Standards GmbH) supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS) and 0.5 μg/ml gentamicin (PAA Laboratories GmbH). Cells were grown until 60–70% confluency and detached by using 0.25% (w/v) trypsin-0.5 mM EDTA solution for sub-culturing.

Table 1
Oligonucleotides used in this study.

Oligonucleotide	Sequence 5′–3′
pksP1707_for	CATCAGTGGTGGTGTAAACC
pksP4745_rev	GCGATAATGTCATCCCCTTC
pksP2744ptrA_rev	GGCCTGAGTGGCCATCGAATTCGTGAAGACGAACCCAGTCTTG
pksP3723ptrA_for	GAGGCCATCTAGGCCATCAAGC CTTCAGCTCGGCTCACGAAG
ptrA-for	GAA TTC GAT GGC CAC TCA GGC C
ptrA-rev	GCT TGA TGG CCT AGA TGG CCT C

Murine alveolar MH-S macrophages (ATCC: CRL-2019) were cultivated in RPMI 1640 supplemented with 10% (v/v) heat inactivated foetal calf serum (PAA) at 37 °C in 5% (v/v) CO_2.

Deletion of the pksP gene

Isolation and manipulation of genomic *A. fumigatus* DNA and Southern blot analysis were carried out as previously described (Grosse et al., 2008). Partial deletion of the *pksP* gene was achieved by employing a PCR based strategy. Fragments of the *pksP* gene were amplified by PCR using primer pairs pksP1707_for and pksP2744ptrA_rev and pksP3723ptrA_for and pksP4745_rev, respectively (Table 1). By this reaction, overlapping ends to the pyrithiamine resistance cassette were introduced at the 3′-end of the upstream region and at the 5′-end of the downstream region of the *pksP* gene. The *ptrA* resistance cassette was amplified from plasmid pSK275 (Szewczyk and Krappmann, 2010) with primers ptrA-for and ptrA-rev. The final deletion construct was generated by a three fragment PCR employing primers pksP1707_for and pksP4745_rev. All PCR reactions were performed with Phusion High-Fidelity DNA Polymerase (Finnzymes) according to the manufacturer's recommendations. The resulting 5 kb PCR product was used for transformation of *A. fumigatus* CEA17 protoplasts. Pyrithiamine (1 mg/ml, Sigma–Aldrich) resistant transformants that produced white conidia were analyzed for partial deletion of *pksP* by Southern blot analysis. One positive transformant, designated CEA17pksP, was chosen for further analysis.

Phagocytosis assay

A549 cells were seeded on glass cover slips in 24 well plates (5×10^5 cells per well) in triplicates and allowed to grow adherently for 16 h at 37 °C in humidified atmosphere of 5% (v/v) CO_2. The cells were washed with pre-warmed F-12K medium without supplements and challenged with fluorescein-5-isothiocyanate (FITC)-labelled conidia in F-12K medium at multiplicity of infection (MOI) of 5. After incubation for 6, 12 and 24 h at 37 °C in 5% (v/v) CO_2, unbound conidia were washed with pre-warmed supplement free F-12K medium and phagocytosis was stopped by adding ice-cold PBS to the wells. Extracellular conidia were labelled with 0.25 mg/ml calcofluor white/PBS (Sigma–Aldrich) for 30 min at 4 °C in the dark followed by three times washing with PBS. As mammalian cells are impermeable for calcofluor white, only phagocytosed conidia retained their FITC signal, whereas non-phagocytosed conidia displayed the calcofluor white fluorescence. Fixation of cells was carried out with 4% (v/v) Rotifix (Carl Roth) for 15 min at room temperature and washing three times with PBS. The coverslips were mounted onto glass slides for microscopy and examined using a Zeiss LSM 5 Live confocal laser scanning microscope. Two random fields containing at least 100 cells per field of three independent experiments were used for determination the percentage of phagocytosis, which was calculated as the average number of conidia that were phagocytosed per phagocyte of a total of 100 phagocytes.

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